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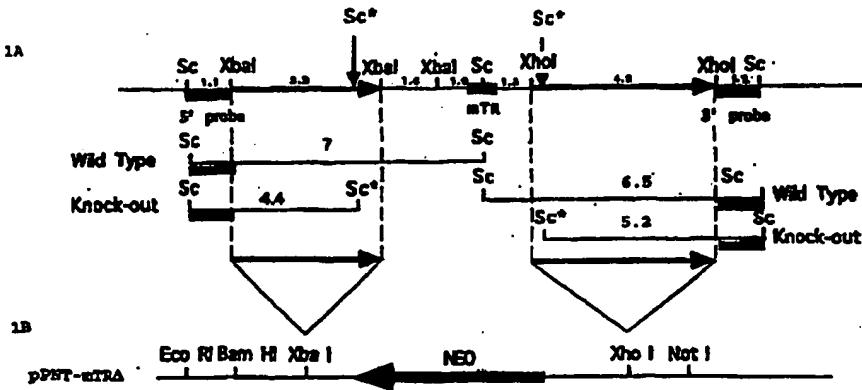
(57) Abstract

Transgenic nonhuman organisms, such as transgenic mice, with altered expression of telomerase are provided. Methods of using these organisms, including methods of detecting compounds that affect the expression of telomerase are also disclosed.

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TRANSGENIC ORGANISMS WITH ALTERED TELOMERASE ACTIVITY

Background of the Invention

Telomerase is a ribonucleoprotein DNA polymerase that adds nucleotide sequence repeats to the telomeres of
5   chromosomes as cells divide. Telomerases are comprised of essential RNA and protein components. Blasco, M.A., et al. (1995) Science 269:1267-1270; Feng, J., et al. (1995) Science 269:1236-1241; Collins, K., et al. (1995) Cell 81:677-686. Without telomerase, the telomeres of
10 chromosomes shorten with each replication until they reach a critical length at which chromosome stability is affected.

Studies of telomerase suggest that the telomerase enzyme may be a new target for cancer therapy and a key to
15 aging and the finite lifespan of somatic cells.   Harley, C., et al. (1990) Nature 345:458-460. Kim, et al. (1994) Science 266:2011-2015, were not able to detect telomerase in a large number of primary cell lines and primary human tissues. When the number of telomeric simple sequence
20 repeats reaches a critical minimum size, these cells become senescent. Harley, C., supra; Counter, et al. (1992) EMBO J. 11:1921-1929. Unlike most somatic cells, germline cells express telomerase and maintain telomere length throughout cell divisions.

25   Short telomeres are also found in immortalized cells, including human tumor cell lines. In contrast to normal human somatic cells, however, cancer cells from tissue culture and those taken directly from tumors contain detectable telomerase activity, suggesting the telomere
30 length is maintained so these cells can divide indefinitely. Counter, et al. (1994) PNAS 91:2900-2904; Kim, et al., supra. These findings suggest that targeting telomerase may be an effective cancer treatment. Harley,

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et al. (1994) *Cold Spring Harbor Lab Symposium on
Quantitative Biology*, Cold Spring Harbor Laboratory, Cold
Spring Harbor, N.Y. Because telomerase plays a key role in
aging and human disorders, it is essential to understand
5 how telomerase functions.

Summary of the Invention

       This invention provides nonhuman transgenic organisms
in which telomerase activity is altered. Organisms with
one altered gene are heterozygous for the alteration;
10 whereas, organisms in which the alteration occurs in both
alleles are homozygous for the alteration.
       In one embodiment, telomerase activity is reduced or
eliminated in the nonhuman transgenic organisms because the
gene or genes encoding one or more telomerase components is
15 "knocked out" (*i.e.*, deleted or otherwise disabled), with
the result that telomerase activity is reduced or
eliminated.
       In a second embodiment, nonhuman transgenic organisms
contain nucleic acid constructs which induce telomerase
20 activity by turning on endogenous genes encoding telomerase
RNA and protein components that are normally silent, or
through insertion of one or more nucleic acid constructs
comprising DNA or RNA encoding telomerase RNA and/or
protein components into the genome of an organism, in which
25 they are expressed. Alternatively, an exogenous DNA
construct, such as a promoter gene, is inserted into the
genome of an organism and alters the normal transcription
or functioning of endogenous telomerase. This promoter can
be an inducible promoter, which is inserted and integrated
30 into the genome of the organism so that endogenous or
exogenous genes encoding telomerase are turned on or off at
particular times and in selected tissues.
       In a third embodiment, a nonhuman transgenic organism
is provided wherein an endogenous telomerase component gene

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is replaced, at least in part, with a telomerase component
gene from another species or a telomerase component gene
from another species which has been altered, thus producing
an organism with a chimeric gene. The replacement can
5   occur on both endogenous genes, resulting in an organism
that is homozygous for the exogenous chimeric gene and is
not capable of expressing any telomerase gene at that
genomic location or is only capable of expressing the novel
nucleotide sequence or transgene.
10   In another aspect, the invention provides a rodent,
such as a mouse in which expression of the gene encoding a
telomerase component is altered. In one embodiment, the
endogenous gene is removed, partially or completely and, as
a result, telomerase activity is reduced or absent. The
15   rodent can be heterozygous or homozygous for the
alteration. In a second embodiment, telomerase activity is
induced by providing a DNA construct which turns on a
normally silent endogenous gene. In a third embodiment,
telomerase expression is altered by insertion, into the
20   genome of the rodent, of a nucleic acid sequence that
replaces all or a part of endogenous DNA encoding a
telomerase RNA or protein component, the result of which is
a chimeric telomerase molecule.
         The nucleic acid sequence which replaces the
25   endogenous gene encoding a telomerase component or a
portion thereof can be a novel DNA sequence, a portion of
the telomerase component gene, a marker gene, a promoter or
other regulatory sequence, or a combination of these
sequences.
30   The invention further provides constructs,
particularly DNA constructs, useful for producing the
transgenic nonhuman organisms, such as transgenic mice,
described herein. Also provided are nucleic acid probes
which can be used to distinguish DNA of a wildtype
35   (naturally-occurring) organism from DNA of an organism in

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which a portion of an endogenous telomerase component gene
has been replaced with an exogenous DNA sequence. Further,
nucleic acid constructs of transgenic unicellular
eukaryotes such as Tetrahymena sp., or in transfected
5   prokaryotes are disclosed which are useful for production
of telomerase or telomerase components.

In another aspect, this invention provides embryonic
stem cells, somatic cells and tissues of a nonhuman
organism which contain one or more copies of the nucleic
10   acid constructs described herein. This includes cells and
tissues comprising knockout constructs or constructs which
induce telomerase expression. The transgenic organisms can
be used as a source of cells for cell culture.

In a further aspect, the invention provides a method
15   of identifying a drug for stimulating telomerase activity
in a transgenic nonhuman organism, such as a mouse, with
reduced telomerase activity. Alternatively, compounds that
inhibit telomerase activity can be identified and/or tested
for toxicity using a transgenic nonhuman organism to
20   determine if telomerase can be inhibited or suppressed
without detrimental effects to the organism. The drug can
be administered to the organism and a sample of cells or
tissues from the organism can be assayed for telomerase
activity and for toxic side effects.

25   The invention further provides a method of using a
transgenic organism or cells or tissues from the organism
or its descendants to identify the control elements of
immortal cells, such as cancer cells, or to identify the
controlling factors in agents designated for anti-tumor or
30   anti-aging purposes. In addition, agents that stimulate or
restrict these phenomena can be identified and developed
for prophylactic or therapeutic applications through the
use of such transgenic organisms and their descendants.

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Brief Description of the Drawings

    Figures 1A and 1B diagram the targeting construct for knocking out the mouse telomerase RNA component. Figure 1A diagrams the portion of the wildtype mouse genome which
5 includes the mouse telomerase RNA component (mTR). Plasmid pPNT-mTRΔ (Figure 1B) shows the vector used to replace the endogenous 3.9 kb chromosomal segment including the mouse gene for the telomerase RNA component, with a neomycin resistance (NEO) gene.

10   Figure 2 is a restriction map of the genomic mTR gene.

    Figure 3 shows the XhoI and XbaI fragments with introduced SacI (Sc*) restriction sites.

    Figure 4 shows the XhoI and XbaI genomic fragments with inserted SacI* (Sc*) sites cloned into the Bluescript
15 SK- and KS+ plasmids and into pPNT-mTRΔ.

    Figure 5 is the nucleotide sequence (SEQ ID NO:1) of DNA encoding the human telomerase RNA component with the telomeric repeat template underlined and the start (ST) and end (STP) of transcription sites marked.

20   Figure 6 is the nucleotide sequence (SEQ ID NO:2) of DNA encoding the mouse telomerase RNA component with the telomeric repeat template underlined.

    Figure 7A-7B is a comparison of DNA encoding the human (hTR), mouse (mTR), rat (rTR) (SEQ ID NO:3), hamster (cTR)
25 (SEQ ID NO:4), and bovine (bTR) (SEQ ID NO:5) telomerase RNA components showing the conserved sequences between the molecules.

    Figure 8 is the nucleotide sequence (SEQ ID NO:6) of the Tetrahymena 80 kD telomerase protein component gene.
30 The nucleotide sequence is derived from genomic and cDNA clones.

    Figure 9 is the amino acid sequence (SEQ ID NO:7) of the Tetrahymena 80 kD protein component deduced from the nucleotide sequence shown in Figure 8.

35   Figure 10 is the nucleotide sequence (SEQ ID NO:8) of

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the *Tetrahymena* 95 kD telomerase protein component gene.

Figure 11 is the amino acid sequence (SEQ ID NO:9) of the *Tetrahymena* 95 kD protein component deduced from the nucleotide sequence shown in Figure 10.

5   Figure 12 is the nucleotide sequence of a genetically-engineered p80 telomerase protein component gene.

Figure 13 is the nucleotide sequence of a genetically-engineered p95 telomerase protein component gene.

Detailed Description of the Invention

10   This invention provides transgenic nonhuman organisms, such as transgenic mice, in which telomerase activity is altered. In one embodiment, telomerase activity is reduced or absent because one or more of the endogenous genes encoding a telomerase component is lacking or does not

15   encode a functional telomerase component. In this example, all or a portion of the endogenous or wildtype gene has been replaced with an exogenous nucleic acid sequence, i.e., a sequence not normally found in the corresponding wildtype species. A knockout organism is one in which both

20   of the endogenous genes for a telomerase component have been completely disabled. Telomerase activity is absent in a knockout organism; i.e., telomerase activity is absent in somatic and germline cells compared to a wildtype organism of the same species. The transgenic organisms described

25   include heterozygous and homozygous organisms. Heterozygotes include transgenic organisms in which one of the two copies of a telomerase component gene is altered and telomerase activity is the same or is reduced in somatic or germline cells compared to a wildtype organism

30   of the same species.

The invention further comprises cells or tissues of transgenic nonhuman organisms wherein telomerase expression is otherwise altered. For example, transgenic nonhuman organisms are provided in which telomerase activity is

activated through the insertion of one or more genes
encoding a telomerase component. Alternatively, a DNA
construct is incorporated into the genome of the organism
and selectively regulates genes encoding telomerase so that
5 telomerase activity can be induced or inhibited at
particular times or in selected tissues.

Transgenic nonhuman organisms are also provided
wherein the organism contains all or part of an exogenous
DNA sequence encoding a telomerase component gene from
10 another species. The resulting telomerase molecules
comprise at least part of an exogenous telomerase component
which replaces the corresponding nucleotide sequence of the
endogenous gene.

As used herein, the term "transgenic nonhuman
15 organism" means organisms which result from alteration of
one or more endogenous genes encoding a telomerase
component (founder organisms) and all subsequent
generations. The term "descendants" refers to any and all
future generations derived or descending from a "founder"
20 transgenic organism, e.g., an organism containing an
exogenous construct as part of its genomic DNA and able to
transmit this construct through its germ cells. Thus,
descendants of any successive generation are included
herein if the descendants contain the alteration or
25 transgene as part of their genome.

The organisms referred to in this application include
all vertebrate and invertebrate multicellular organisms
described in the kingdom Animalia, unicellular and
multicellular fungi, and all animal-like protists. The
30 term "animal-like protists" includes all unicellular
eukaryotes that are absorptive or ingestive heterotrophs,
including, e.g., Tetrahymena sp., Amoeba, sp., and
parasitic protozoa such as Trichomonas sp., Giardia sp.,
Entamoeba sp., Plasmodium sp. and Leishmania sp.. See,
35 e.g., Lee, J.J., et al. (eds.) An Illustrated Guide to the

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Protozoa, Society of Protozoologists, Lawrence KS, 1985.
All references to nonhuman organisms are meant to include
species of Animalia, Fungi, and animal-like species of the
Protista, except in instances where it is obvious that a
5 multicellular animal is intended (*e.g.*, references to
blood, vertebrate, ES cells, an organ, tumor, etc.).

Although mice homozygous for the deletion of a
telomerase component (homozygous null) are known (see
Example 6), cells or tissues can be derived from any stage
10 of development of an organism homozygous for a telomerase
deletion, such as a homozygous embryo, fetus, or immature
animal. The cells can be cultured, using standard cell or
tissue culture techniques, and then used to study the
functioning of cells in the presence of various agents and
15 can function as implants in organisms with normal
telomerase activity.

Implants can be especially useful when inserted into
nude mice which are prone to tumor formation. The mice,
for example, can be exposed to carcinogens and the knockout
20 tissues observed to determine if tumor formation occurs in
the absence of telomerase. Therefore, the present
invention provides a method to determine the relationship
between telomere shortening, telomerase activity and tumor
formation.

25 As an alternative, an exogenous gene encoding a
telomerase component can be combined with an inducible
promoter and integrated into the genome of a knockout
organism of the present invention so that telomerase
expression can be turned on or off at certain times or in
30 particular tissues of the transgenic organism. Also, a
"conditional knockout" can be generated (Example 8) if a
knockout transgenic organism has a significantly reduced
viability, making it difficult to breed, or if the absence
of telomerase in all tissues results in additional
35 phenotypes that complicate studies of the effects of

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telomerase deletion. In one embodiment, a mouse is
produced that is heterozygous for the mTR knockout while
the endogenous mTR gene is flanked by a recombination site
on either side of the gene. When the mouse is bred to a
5   mouse with a gene encoding recombinase, most of the cells
of the F1 mouse have one copy of the recombinase. Thus, in
selected tissues, telomerase expression is completely
knocked out.
        The alternative transgenic nonhuman organisms
10 described above are useful if a knockout embryo of an
animal species other than a mouse cannot survive.
Telomerase transcription in the knockout can be turned off
at any stage of development to ascertain when and where
telomerase is essential, especially in growth and aging
15 processes, or in tumor formation. Alternatively,
telomerase transcription in an adult animal can be turned
on or off in selected tissues so that the effects of a drug
can be determined with and without telomerase activity.
        Modifications of organisms through transgenic
20 procedures can produce telomerase alterations of various
types, including insertions, deletions, substitutions, or
additions of nucleic acids or amino acids, or any
combination of the preceding. A specific example of such a
modification is the inactivation of the telomerase RNA
25 component gene by site-specific integration of a nucleotide
sequence that replaces the endogenous gene encoding the RNA
telomerase component of the mouse, as described in the
Exemplification. Using this technique to knock out a gene
by gene targeting avoids problems associated with the use
30 of antisense RNA to disrupt functional expression of a gene
product. As described in Examples 2 and 3, a selectable
marker gene, flanked by DNA sequences isogenic to the
sequences at the 5' and 3' most ends of the gene segment to
be replaced, is inserted into the exogenous DNA construct
35 so that homologous recombination between the exogenous

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construct and the endogenous target DNA results in
insertion of the selectable marker gene into a coding
region or essential regulatory element of the target gene.
The term "selectable marker gene" refers to a nucleic acid
5   sequence whose expression allows for selection of targeted
cells that have stably incorporated the exogenous DNA,
making it possible to screen the targeted cells or
derivatives of these cells for heterozygosity.

Examples of genes encoding selectable markers include,
10   but are not limited to: genes conferring resistance to
compounds such as antibiotics, genes conferring the ability
to grow on selected substrates, and genes encoding proteins
that produce detectable signals, such as dye staining or
luminescence. A wide variety of such markers are known and
15   available, including, for example, antibiotic resistance
genes such as the neomycin resistance gene (NEO), Southern,
P. and Berg, P. (1982) *J. Mol. Appl. Genet. 1:327-341*
(1982); and the hygromycin resistance gene (HYG) Kaster,
K., et al. (1983) *Nucleic Acids Res. 11:6895-6911*, and Te
20   Riele, H., et al. (1990) *Nature 348:649-651*. Other
selectable markers for use in organism cells, and plasmids
carrying a variety of genes encoding selectable markers,
are described in Sambrook, J., et al. *Molecular Cloning: A
Laboratory Manual*, Cold Spring Harbor Laboratory, Cold
25   Spring Harbor, N.Y. (1989).

The invention also provides embryonic stem (ES) cells
and embryonic stem cell lines in which an endogenous
telomerase component gene has been knocked out (deleted or
otherwise disabled) by the methods described herein. A
30   further subject of the invention are ES cells and ES cell
lines in which a telomerase component gene has been
replaced with a different gene. The replacement gene can
encode a chimeric product; for example, a gene encoding a
mouse/human telomerase RNA component wherein the human
35   template sequence replaces the mouse template sequence in a

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mouse telomerase RNA component gene. Such a construct is
useful to determine if human telomerase can be produced and
are capable of elongating the telomeres of mouse
chromosomes. A replacement gene can also encode a complete
5 or functional portion of a telomerase RNA or protein
component sequence from another organism species. Several
examples of genes encoding telomerase RNA or protein
components are shown in Figures 5, 6, 7A-7B, 8, and 10.
Active telomerase comprising chimeric combinations of RNA
10 and protein components of different species can provide
valuable information concerning phylogenetic origins and
can also provide therapeutic models for recombinant
molecules that can be used in gene therapy. It could
further be determined if a construct for replacement of the
15 RNA component gene which retains the conserved nucleotides
of the genes encoding telomerase RNA component of different
species, as shown in Figure 7A-7B, can produce active
telomerase in any organism.

Transgenic organisms in which the gene encoding
20 telomerase can be turned on or off in cells or tissues are
useful for studying aging and control of tumor growth, as
well as many other growth processes. For this purpose,
chimeric sequences which comprise an inducible promoter
coupled to a telomerase component gene can be used to
25 increase or decrease telomerase activity in particular
cells and tissues at preferred times. For example, the
tetracyline-responsive cytomegalovirus promoter effectively
allows differential control of the activity of genes in
mammalian cells. Gossen, M. and Bujard, H. (1992) *Proc.*
30 *Natl. Acad. Sci. USA 89*:5547-5551. Cells and tissues
comprising telomerase component genes under such controls
are useful in the study of cellular senescence and
immortalization.

The capability to maintain chromosomal replication
35 (*i.e.*, cell division) has many therapeutic possibilities,

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among which is gene therapy. During gene therapy, cells
are normally extracted from individuals, the genome of the
cell is altered to correct a genetic defect or produce a
required protein (such as erythropoietin), and the altered
5   cells returned to the individual. If immortalized cell
lines are not used, the extracted cells are limited in
their number of cell divisions so that small numbers of
therapeutic cells are produced. Instead of using
immortalized cell lines, which raises the possibility of
10   tumor induction, blood or tissue cells of an individual can
be genetically modified to incorporate genes encoding
telomerase as well as nucleic acids encoding a therapeutic
protein or proteins. The encoded telomerase can lengthen
the number of cell divisions, thus lengthening the life
15   span of the modified cells prior to therapy or after
introduction into an individual. Larger numbers of
therapeutic cells could be produced for delivery to the
individual. (The individual from which the cells were
obtained or another individual.)   The prophylactic and
20   therapeutic possibilities are not limited, and include any
application wherein the control of cell division, is
beneficial to the organism.

A replacement sequence for an endogenous telomerase
gene, e.g., the sequence diagrammed in Figure 1B, can
25   encode a marker, such as a neomycin resistance gene. The
marker gene can be coupled to a promoter which is
constitutive or inducible. It is expected that the
expression of the marker will be used to detect cells
wherein the marker DNA sequence has replaced an endogenous
30   telomerase component sequence and homologous recombination
has occurred.

This invention also includes constructs, particularly
DNA constructs, useful for producing the transgenic
nonhuman organisms, such as the transgenic mice, described
35   herein. The DNA comprising the knockout or replacement

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construct will usually include one or more exon(s) and/or
intron(s), or regions thereof of a gene encoding a
telomerase component, and/or a promoter region. Any DNA
that is the functional equivalent of a telomerase component
5 can be used. The functional equivalent of a telomerase
component gene is a gene encoding a molecule that is
capable of combining with other endogenous or exogenous
telomerase components to form an active telomerase enzyme.
Other DNA can comprise promoters, sequences which encode
10 markers, and modified or synthetic gene sequences.

     Generally, the DNA construct will be at least about 1
kilobase (kb) in length and preferably 3-15 kb in length,
thereby including sufficient complementary sequence for
recombination when the exogenous construct is introduced
15 into the genomic DNA of the targeted cell. Larger
constructs may be required if the replacement is comprised
of several genes and promoters or regulatory sequences.
The preferred genes to be altered are any or all of the
genes encoding the RNA component and the protein
20 component(s) of telomerase.

     There may be one or more protein components comprising
the telomerase enzyme, depending on the species of cell
targeted. The *Tetrahymena* telomerase ribonucleoprotein,
for example, contains a 95 kb protein (p95), an 80 kb
25 protein (p80) (Figures 9 and 11, respectively), and a 159
nucleotide RNA component. The RNA component contains a
short internal sequence which serves as a template for
synthesis of the G-rich strand of a telomeric repeat. The
two protein components have different nucleic acid binding
30 properties: p95 binds specifically to telomeric primer
DNA, whereas p80 binds most specifically to the telomerase
RNA.

     Both the RNA component and the protein component of
telomerase are essential for enzymatic activity.
35 Therefore, it is only necessary to ensure that one of the

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telomerase components, either the RNA component or a
protein component, is not transcribed or that the product
of the gene is not functional to diminish or suppress the
activity of telomerase. As used herein, the term
5   "nonfunctional" refers to the inability of the expressed
component to combine with any of the other telomerase
components to form an enzyme capable of adding telomeric
repeats to chromosome ends or to the inability to perform
its specific enzymatic role once combined.
10   The DNA sequence used in producing the knockout
construct is digested with a particular restriction enzyme
selected to cut at a location such that a new DNA sequence
encoding a marker gene can be integrated in a position
within this DNA sequence and transcription of the
15   endogenous gene will be prevented after insertion of the
knockout construct into the chromosome. Those of skill in
the art will recognize the various factors that will affect
the selection of useful restriction sites for this purpose.
The marker gene to be inserted will normally have a polyA
20   addition site attached to its 3' end. The marker can be
operably linked to its own promoter or to another strong
promoter from any source that is active or can easily be
activated in the cell into which it is integrated.
Alternatively, the marker gene can be transcribed using the
25   promoter of the gene that is suppressed.
         By flanking the exogenous gene of the construct with
sequences substantially isogenic with the target DNA in the
host cell, it is possible to introduce the gene in a site-
specific fashion at the targeted location. Using this
30   approach, a gene from any source (e.g., bacterial, fungal,
plant, animal) can be introduced into the organism host
cell to impart new telomerase characteristics to the cell,
replace a telomerase component, or otherwise alter the cell
to produce desired telomerase products which can then be
35   used to assay in vivo for the effects of new drugs.

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Isolated cells and tissues from the organism or from its
descendants can also be used for assays *in vitro*.

An example of a knockout construct of the present
invention is shown in Figures 1B and 4. The plasmid pPNT-
5   mTRΔ comprises the selectable marker gene for neomycin
resistance inserted in a position where it will replace the
endogenous gene which encodes the RNA component of mouse
telomerase. See Figure 1A. The targeted endogenous
sequence to be deleted comprises a 3.9 kb segment which
10 includes the gene encoding the mouse telomerase RNA
component (mTR), and is located between a 3.3 kb *Xba*I
fragment at the 5' end and a 4.0 kb *Xho*I fragment at the 3'
end of the endogenous sequence.

The replacement sequence is ligated into the genomic
15 DNA sequence after the genomic DNA sequence has been
digested with the appropriate restriction enzymes. To make
the ends of the fragments compatible for ligation, the ends
can be blunted, for example by Klenow fragment, or all
fragments can be cut with enzymes that generate compatible
20 ends. Methods for carrying out these procedures are well
known to those skilled in the art and can be found in
Sambrook *et al.*, *supra*. The ligated construct can be
inserted directly into ES cells or it can be incorporated
into a suitable vector for amplification prior to
25 insertion.

Transgenic animals from any species of rodent,
including without limitation, rabbits, rats, hamsters, and
mice, can be produced, as can other nonhuman transgenic
organisms, such as dog, cat, pig, sheep, cow and primates.
30 In most cases, the ES cells used to produce the transgenic
animal will be of the same species as the transgenic animal
to be generated. Thus, for example, mouse embryonic stem
cells will usually be used for the generation of knockout
mice. Transgenic animals can be prepared using methods
35 known to skilled artisans. See, for example, Hogan, *et al.*

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(eds.), *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1986).

The ES cells can be selected for their ability to
5   integrate into and become part of the germ line of a developing embryo so as to result in germline transmission of the inserted DNA construct. Thus, any ES cell capable of such integration is suitable for use herein.

Methods of insertion of DNA vectors into ES cells
10 include, but are not limited to, electroporation and microinjection. The insertion of DNA constructs into ES cells by electroporation is described in Example 3 of the Exemplification.

ES cells can be screened for the inserted construct by
15 a variety of methods, which can be used alone or in combination. If a marker sequence has been incorporated, the appropriate conditions and procedures are applied to identify the marker product in the cells as an indication of the presence of the construct. For example, where the
20 marker is an antibiotic resistance gene, such as the neomycin resistance gene, the cells are contacted with a concentration of the antibiotic (neomycin) which is lethal to cells which do not express a functional neomycin resistance gene. Those cells that survive are presumed to
25 have integrated the construct into their genome because they are resistant to neomycin, whereas the wildtype cells are not. Or, the marker gene can encode an enzyme whose activity is detected by contacting the cells with the appropriate substrate for the encoded enzyme, then assaying
30 for enzyme activity (or product).

Alternatively, or in addition, a Southern blot of the genomic DNA of the ES cells can be probed with a DNA sequence which hybridizes to the marker sequence. Probes such as the 1.1 kb and 1.2 kb probes shown in Figure 1A and
35 2, can be used to distinguish the genome of ES cells or

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tissues of transgenic organisms which are either unchanged
or in which the inserted construct has randomly integrated
into the genome from those in which the knockout construct
has integrated into targeted site (mTR) of the genome.

5   These probes are especially useful to determine if the
germline cells of the transgenic organism include a
knockout construct for the telomerase RNA component.

        Figure 1A illustrates a 1.1 kb probe at the 5'
flanking end of the wildtype chromosome and a 1.2 kb probe

10 at the 3' flanking end of the same chromosome, either of
which can be used to determine if the knockout construct of
pPNT-mTRΔ has replaced the endogenous mouse telomerase RNA
component gene through homologous recombination. The 5'
flanking probe is located between SacI (Sc) and XbaI; the

15 3' flanking probe is located between XhoI and SacI.
Following digestion with enzyme SacI, the fragment lengths
of DNA isolated with these probes will be shorter after
excision from the knockout construct due to the insertion
of a unique SacI (Sc*) restriction site engineered into the

20 XbaI and XhoI genomic fragments which are incorporated into
the knockout construct (see Example 2). As shown in Figure
1A, the wildtype gene will produce a 7.0 kb fragment
including the 5' probe and a 6.5 kb fragment with the 3'
probe. If the NEO gene has replaced the mouse telomerase

25 gene, a 4.4 kb fragment (5' probe) or a 5.2 kb fragment (3'
probe) will be produced. Therefore, if the 5' most probe
is used for detection, wildtype cells will produce only a
7.0 kb band; a heterozygous cell will produce 7.0 kb and
4.4 kb bands, and those cells homozygous for the knockout

30 construct (homozygous null) will produce a 4.4 kb band
only. In a similar manner, the bands detected with the 3'
most probe will be 6.5 kb (wildtype cells), 6.5 kb and 5.2
kb (heterozygous cells), or 5.2 kb (homozygous null cells).
Those of skill in the art will recognize that other probes

35 can be isolated and used with these methods to determine if

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replacement of a telomerase component gene has occurred in
cells and tissues of a transgenic organism.

The ES cells carrying a knockout or altered construct
are introduced into embryos using known methods. For
5   example, they can be microinjected into eggs according to
known protocols, such as those described in Example 5.
Other methods for production of transgenic rodents are set
forth in Hogan, et al. (eds.), *Manipulating the Mouse
Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory
10 Press, Cold Spring Harbor, N.Y. (1986).

The developing ES cell carrying the construct is
injected into a blastocyst and implanted into a
pseudopregnant female of an appropriate strain and allowed
to develop into offspring. As an option, the blastocyst
15 can be fertilized and incubated *in vitro* prior to
introduction into the female.

Following implantation, gestation, and birth, the
nonhuman organism which develops from the embryo is a
transgenic nonhuman organism, preferably one in which the
20 germline cells contain the altered construct. These
founder organisms, which are likely to be heterozygous for
the altered construct, can be bred to produce homozygotes.
See Example 6. Heterozygotes, as well as being useful to
generate knockout organisms, can be altered to produce
25 further genetic modifications affecting telomerase activity
resulting from the expression of an endogenous telomerase
component gene. Conditional knockout organisms as
described in Example 7 represent one application of a
modification.

30   The transgenic organisms and cells derived from such
organisms have a variety of uses, which depend on the
telomerase component expressed and their integrated
constructs. Such organisms can be used to screen for drugs
or a therapeutic regimen useful for prophylactic or
35 therapeutic treatment of diseases such as cancers and for

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the regeneration of cells and tissues that do not normally
divide infinitely.

    More specifically, this invention provides a method of
identifying an agent that is active in stimulating
5 telomerase activity, which comprises:
        a)   administering an agent to a transgenic nonhuman
              organism, wherein the organism does not express
              telomerase activity or has diminished telomerase
              activity but has the capability to produce
10   telomerase; and
        b)   assessing the effect of the agent on the
              telomerase activity,
    wherein if the agent causes an increase in telomerase
activity, the agent is active in stimulating telomerase
15 activity.

    This invention further provides a method of
identifying an agent that is active in stimulating
telomerase activity, which comprises:
        a)   administering an agent to a sample of transgenic
20   cells or tissue, wherein the sample does not
              express telomerase activity or has diminished
              telomerase activity but is capable of producing
              telomerase activity; and
        b)   assessing the effect of the agent on the
25   telomerase activity,
    wherein if the agent causes an increase in telomerase
activity, the agent is active in stimulating telomerase
activity.

    Additionally, this invention provides a method of
30 identifying an agent that is active in inhibiting
telomerase activity, which comprises:
        a)   administering an agent to a transgenic nonhuman
              organism, wherein the organism expresses
              telomerase activity; and
35   b)   assessing the effect of the agent on the

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telomerase activity,
wherein if the agent causes a decrease in telomerase
activity, the agent is active in inhibiting telomerase
activity.

5   The method can also be used with samples of transgenic
cells or tissue which express telomerase and are derived
from transgenic nonhuman organisms to determine if the
agent inhibits telomerase activity.

        Just as the organisms of this invention can be used as
10 models to study telomerase activity, they can also provide
a system in which a material suspected of being a
carcinogen can be tested by exposing the organism to the
material and determining neoplastic growth as an indicator
of carcinogenicity. Transgenic nonhuman organisms, in
15 particular, are very useful to develop effective therapies
or regimens for combatting diseases or conditions and to
ascertain the overall specific effects of a drug on
telomerase activity in a living system.

        The role of telomerase in the development and growth
20 of tumors can be determined by comparing the effects of
carcinogens and other tumor-inducing mechanisms in tissues
wherein telomerase activity is turned on or off. See,
e.g., Examples 7 and 8. Telomerase activity in tumor cells
maintains the severely-shortened telomeres of these cells.
25 The absence of telomerase in tumor-forming cells could
retard and even stop the growth of tumors if the dividing
tumor cells lose their telomeres completely due to lack of
telomerase activity.

        Another important feature of the present invention is
30 that it provides, for the first time, an organism wherein
the side effects of drugs affecting telomerase activity can
be ascertained and distinguished from the effects of
diminished or enhanced telomerase activity. By "side
effects" it is meant those effects of a drug which are not
35 attributable to the alteration in telomerase activity.

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Further, the side effects of such drugs as they affect the
descendants of treated organisms can be determined. In
particular, the toxicity of telomerase activity inhibitors
can be tested *in vivo* using mammalian systems such as the

5   transgenic mice described herein. For example, genes
encoding human telomerase can be used to replace endogenous
telomerase genes in mice, and the effects of telomerase
inhibitors can be studied in a mammalian system over
several generations so that any short-term or long-term

10 side effects of the inhibitors can be documented along with
the specific effects of alterations in telomerase activity.
In addition, screening of these drugs for their effects on
particular transgenic cells and tissues can be done *in vivo*
or *in vitro*.

15   More specifically, this invention provides a method of
identifying side effects of an agent that modifies
telomerase activity in an organism, which comprises:

    a)   administering the agent to a transgenic nonhuman
        organism which is incapable of expressing

20   telomerase; and

    b)   assessing the effects of the agent on the
        transgenic nonhuman organism,

wherein if the agent causes one or more effects on the
organism, it is identified as an agent that causes one or

25 more side effects.

In knockout organisms or tissues, shutting down the
production of telomerase may result in the inability of
cells to immortalize, thus, making tumor formation
impossible. In addition to using knockout organisms for

30 demonstration of these effects, cells or tissues comprising
the knockout construct can be transplanted onto nude mice
which have a propensity to form tumors. The absence or
reduced incidence of tumors at the transplantation sites
would show that telomerase is required for tumor formation.

35   Alternatively, conditional knockout organisms or those

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in which a telomerase component is under the control of an
inducible promoter, as described *supra*, can be used to
induce suppression of telomerase production either before
or after tumor formation. It could then be determined if
5   tumor formation can occur in the absence of telomerase and
if suppression of telomerase causes cell division to cease
in tumors.

    In particular, mouse model systems for tumor formation
have generated a wealth of information about cancer
10 progression. Palmiter, R.D. and Brinster, R.L. (1985) *Cell*
41:343-345; Cory, S. and Adams, J.M. (1988) *Ann. Rev.*
Immunol. 6:25-48; Hanahan, D., et al. (1989) *Science*
246:1265-75. The expression of oncogenes in transgenic
mice provided definitive evidence that oncogenes cause
15 tumors in mammals. Further, the finding that not all cells
that express these oncogenes form tumors supported the
multiple hit model for tumor progression. Brinster, R.L.,
et al. (1984) *Cell 37*:367-379; Adams, J.M., et al. (1985)
Nature 318:533-538; Hanahan, D. (1985) *Nature 315*:115-122;
20 Cory and Adams, *supra*. Mouse models for tumor suppressor
genes have been developed by creating homozygous germline
deletions in mice. Donehower, L.A., et al. (1992) *Nature*
356:215-221; Jacks, T., et al. (1992) *Nature 359*:295-300;
Jacks, T., et al. (1994) *Curr. Biol.* 4:1-7; Jacks, T., et
25 al. (1994) *Nature Genet.* 7:353-361. Mice that are
homozygous null for a telomerase component, such as the mTR
(-/-) mice, or heterozygous mTR (+/-) mice can be crossed
to both oncogene expressing mice and mice deleted for tumor
suppressor genes, and the mTR (-/-) oncogene-expressing
30 offspring examined to determine if the absence of
telomerase results in a lower rate of tumor formation,
smaller tumor size or a lower frequency of metastasis.
Further, a recombination deficient mouse can be used to
generate a mTR (-/-) mouse into this background to look at
35 the effects of recombination telomerase bypass pathways.

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See Example 8.

An organism or tissue system in which telomerase
activity is under the control of an inducible promoter, can
also be used to study senescence. Most somatic cells lose
5   segments of their telomeres as they divide in culture;
whereas, telomeres are maintained in germline cells.
Greider, C. and Blackburn, E.H. (1996) *Scientific American*
274(2):92-97. Stimulation of telomerase activity in
somatic cells of an aging transgenic nonhuman organism
10   could answer many questions about the role of telomerase
during periods of cellular senescence, especially the
effects of telomerase in atherosclerosis or the decline in
immunity individuals experience as they age.

Those skilled in the art will also recognize the value
15   of the organisms, cells and tissues of this invention as a
tool for understanding the growth and division of cells,
and for production of cells and tissues that, in their
wildtype form, are difficult to study *in vitro* because they
undergo a small number of cell divisions.

20   EXEMPLIFICATION
                               Example 1
Map of mTR Genomic Locus

A 15 kb lambda genomic clone containing the mouse
telomerase RNA gene was subjected to restriction enzyme
25   digestion and various resultant fragments were subcloned
into a Bluescript vector for further analysis. To map the
different genomic fragments, Southern blots were probed
with a 2 kb genomic fragment containing the mTR gene.
Restriction enzyme sites are shown for the following
30   enzymes; *SacI, XhoI, EcoRI, XbaI, EcoRV, BamHI,* and *PstI*
(Figure 2).

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Example 2

Construction of the Targeting Vector

       To generate a mTR genomic deletion construct, site-
directed mutagenesis was used to introduce two new unique
5   SacI restriction sites (SacI* or Sc*) into the 3.3 kb XbaI
and 4.0 kb XhoI genomic fragments (Figure 1A). These
unique sites were engineered so that the correct homologous
recombination events in ES cells could later be identified
by digestion of genomic DNA with SacI. See Figures 1A and
10   4. Using a 1.2 kb probe (mTR probe) to the right of the
mTR gene following digestion with SacI yields a 6.5 kb
wildtype band or a 5.2 kb band from the correctly targeted
deletion allele. The probe was labeled by random primer
extension using Klenow enzyme, ^{32}P-dGTP (3000 Ci/mmol),
15   ^{32}P-dATP (3000 Ci/mmol) and cold dCTP and dTTP.
       To introduce the SacI* sites, both the XbaI and XhoI
genomic fragments were cloned into Bluescript SK- and KS+,
respectively (Figures 3 and 4), and the orientation of the
clones was determined by restriction enzyme digests.
20   Approximately 250 nt of the 3' end of XbaI fragment and the
5' end of the XhoI fragment were sequenced and
oligonucleotides were designed to introduce the SacI* sites
by site-directed mutagenesis.
       The engineered SacI* restriction sites were
25   introduced into a subcloned XbaI 4.0 kb fragment located 5'
of the transcribed region and a subcloned 3.3 kb XhoI
fragment located 3' of the transcribed region. The
creation of these restriction sites was confirmed by
sequencing and restriction enzyme digests. After the
30   mutagenized fragments were sequenced, they were cloned into
the targeting vector pPNT (Tyulewicz, V.L., et al. (1991)
Cell 65:1153-1163) to generate the plasmid pPNT-mTRΔ (See
Figures 1B and 4). This vector contains a Neomycin
resistance gene for positive selection of resistant clones
35   and the HSV-tk gene (Thomas, K.R. and Cappechi, M.R. (1987)

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Cell 51:503-512) for counter selection in gancyclovir of
incorrectly integrated constructs.

The arrangement of the two mTR genomic fragments in
the targeting construct pPNT-mTRΔ is such that when
5   homologous recombination occurs after electroporation of
mouse ES cells, the mTR gene is replaced by the Neomycin
resistance gene (NEO).

Example 3
Electroporation of pPNT-mTRΔ into WW6 ES Cells

10   WW6 ES cells (E. Ioffe, et al. (1995) *Proc. Natl.
Acad. Sci. 92*:7357-61) were thawed and grown in ES medium
with Leukemia Inhibitory Factor (LIF). The plasmid pPNT-
mTRΔ was linearized with the restriction enzyme *Not*I, and
the DNA was electroporated into WW6 cells by standard
15 protocols. Ramirez-Solis, R., et al. (1993) *Meth. Enzymol.
225*:855-929. Cells were selected in G418 (100 μg/ml) and
gancyclovir (2 μM), and positive clones were picked after
9-10 days growth into duplicate 48 well microtitre plates;
one for DNA analysis, the other for both freezing at -80°
20 C. and potential blastocyst injection. Cells in DNA plates
were grown to near confluency, harvested in Proteinase K
solution (0.1% SDS, 0.5 mg/ml of Proteinase K), and
analyzed for homologous recombination.

Example 4
25 ### Southern Analysis of Doubly-Resistant ES Cell Clones

Genomic DNA was prepared from over 400
G418/gancyclovir resistant cells grown in one well of a 48
well-plate after digestion with Proteinase K solution for
one hour at 37° C. followed by phenol extraction and
30 isopropanol precipitation. Approximately 20 μg of DNA was
digested overnight with 40 units of *Sac*I, in the presence
of RNase A. After digestion, samples were loaded on a 0.8%
agarose/1X TBE gel containing Ethidium bromide and

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subjected to electrophoresis for 24 hr at constant voltage
(30 volts). For Southern analysis, the gels were denatured
and neutralized following standard protocols and
transferred to a nylon filter (Hybond N+ Amersham). After
5   transfer, filters were washed in 2X SSC and the DNA was UV-
crosslinked to the membrane using a Stratalinker. Filters
were pre-hybridized in high stringency solution (1% bovine
serum albumin, 200 mM sodium phosphate, 15% formamide, 1 mM
EDTA and 7% sodium dodecyl sulfate) at 65° C. for two
10 hours. Hybridization with an mTR probe (described below)
was carried out overnight in the same solution. After
hybridization the blots were washed in 0.2X SSC and 0.1%
SDS at 65° C. and exposed to autoradiographic film.

Example 5

15 ## Generation of Chimeric and Heterozygous Mice Deleted for
mTR
        Of the 400 Neomycin/gancyclovir resistant clones
initially screened, four were found by Southern blot
hybridization to contain one correctly targeted mTR locus
20 that was deleted for the mTR coding region. These four
clones were designated Tel-1, Tel-2, Tel-3 and Tel-4. Each
clone was thawed from the frozen 48 well-plate and grown in
culture before injection into C57BL/6J blastocysts. Three
of the clones (Tel-1, Tel-2, and Tel-3) were independently
25 injected and the blastocysts were implanted into pseudo-
pregnant mice. (See Table 1 for numbers of injected mice).
The chimeric mice which were born from these mothers were
identified by their mosaic coat color (resulting from the
agouti contribution of the ES cells and the black
30 contribution of the blastocysts). The mice generated from
the Tel-1 microinjections had over 90% ES contribution from
the injected WW6 cells, based on the percent of agouti
versus black coat color.
        These chimeric mice were then mated to C57BL/6J to

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test for germline transmission of the knocked out allele.
The number and sex of chimeric and heterozygous mice from
each injection are shown in Table 1. Tail DNA from the
progeny of this cross was analyzed by the Southern blot

5 procedure described above using the unique 1.2 kb genomic
mTR probe. Heterozygous mTR (+/-) mice having one wild
type allele and one knockout allele were considered to have
germline transmission for the targeted mTR (+/-) WW6 ES
cells.

10   Example 6
Generation of Mice Homozygous null for mTR
        Homozygous knockout mice can be generated by three
methods:
        a)   Initially a male chimeric mouse which has

15 previously exhibited germline transmission is mated to a
heterozygous female mTR knockout mouse. If telomerase null
mice are viable, up to 25% of the progeny born from this
cross are expected to be mTR (-/-).
        b)   Homozygous mTR mice are also generated by

20 directly crossing sibling male and female heterozygotes
(mTR (+/-) sibling mating). In this cross, 25% of the
progeny are expected to be mTR (-/-).
        c)   ES cells homozygous null for mTR can be generated
from intrachromosomal homologous recombination at increased

25 concentration of G418 (higher than 1 mg/ml). These
homozygous null ES cells are injected into C57BL/6J
blastocysts and transferred to the uteri of pseudopregnant
females. Resultant chimeric mice contain varying ES-
contribution to somatic tissues. An inert molecular tag

30 permits an assessment as to whether cells are derived from
ES or host blastocysts.
        The method described in b) was used to produce
homozygous null mice. See Table 1.

Table 1

Production of Telomerase-Deficient Mice

Telomerase (+/-) ES Cell Clone	Number of Blastocysts injected	Number of Chimeras (%Chimerism)	Number of Germline Chimeras	Number of (+/-) Animals	Litters from (+/-) x (+/-)			
					#	(+/+)	(+/-)	(-/-)
Tel-1	124	5♂ + 6♀ (60-95%)	4♂ + 1♀	12♂ + 12♀ DOB 9/29 ~10/18/95	A	2	5	0
					B	0	3	0
					C	2	3	1
					D	1	3	0
					E	3	5	0
					F	0	3	0
					G	2	3	1
					H*	3	5	1
Tel-2	53	6♂ + 4♀ (40-80%)	mated					
Tel-3	134	9♂ + 3♀ (60-100%)	mated					
Tel-4	not injected							

* Embryos were taken prior to birth and used for generation of fibroblast cultures.

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A total of 8 transfections were performed with
approximately 2×10^7 or 4×10^7 WW6 ES cells per
transfection. Six transfections with an average enrichment
of 4.50 provided 158 clones which were analyzed by Southern
5   blot, demonstrating one heterozygous null (+/-) cell line
(Tel-1). The average enrichment for two other
transfections (where 4×10^7 cells were electroporated),
was 5.75. Three (+/-) ES clones (Tel-2, Tel-3 and Tel-4)
were isolated among 280 clones screened. In summary, the
10   homologous recombination event of telomerase occurred at
0.17% (four in 2321 clones) in WW6 ES cells. The low
frequency of homologous recombination made it difficult to
obtain mutant ES clones. Therefore, efficient subcloning
and screening strategies were developed which permitted a
15   more rapid and extensive analysis of candidate mutant
clones. These procedures were crucial to the success of
this work. Moreover, a unique ES cell line, WW6, was
important to the success of the gene targeting experiments.
The mixed genetic composition of WW6 provides enhanced
20   capacity to contribute to competent germ cells. Virtually
all of the mutant ES cell clones that have been generated
with this cell line have passed the mutant allele through
the germline.

One heterozygous null (+/-) clone (Tel-1) has
25   transmitted through the germline, producing animals that
are heterozygous null (+/-) for telomerase. Heterozygous
null animals were intercrossed to examine the viability of
homozygous null animals. Because WW6 ES cell line has a
mixed genetic background (75% 129/sv, 20% C57Bl/6J, and 5%
30   SJL), some of the (+/-) animals were crossed with C57Bl/6J
mice to produce the inbred strain of C57Bl/6J.

To generate homozygous knockout mice, heterozygous mTR
(+/-) mice were crossed and the progeny examined by
Southern blotting of tail DNA. Of 46 progeny examined from

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the first eight litters, three homozygous null mTR (-/-)
pups were found (Table 1). The initial screening was done
using *Sac*I cut genomic DNA looking for a 5.2 kb band
diagnostic of the deleted allele in place of the wildtype
5   6.5 kb band (Figure 1A). Several control procedures
confirmed that the mice were null for mTR. Genomic DNA was
cut with *Eco*RI and probed with the coding region for mTR.
The wildtype and heterozygous pup DNA produced a 5.0 kb
band; whereas, this band was absent in the deleted mTR (-/-
10) DNA. PCR was also carried out, using primers in mTR and
just inside the *Xho*I site to the right of the gene. A
correctly-sized band of 1.3 kb was generated with the
wildtype and heterozygous DNA but not with the homozygous
null DNA.
15   Of ten pups born in the first two litters of the mTR
(+/-) intercross, none were homozygous null (2 were (+/+)
and 8 were (+/-)). However, the subsequent litters
produced 24 homozygous null pups out of 93 born (31
homozygous normal and 62 heterozygous), indicating that
20 animals without a gene encoding a telomerase RNA component
can survive to birth. The mTR (-/-) pups are similar to
their littermates in size and appearance. Both cells and
tissues derived from homozygous null mice lack telomerase
activity.

25   Example 7
Generation of Conditional mTR Deletions
        Several techniques for producing conditional knockout
mice are known in tissue culture (Sauer, B. and Henderson,
N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170) and can be
30 modified to produce mice with conditional deletions of
either mTR or a telomerase protein component which are
specific to certain tissues. In one procedure, the genomic
region to be deleted is flanked by Lox P recombination
sites. Lox P sites are DNA sequences recognized by the

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bacteriophage P1 Cre recombinase. Cre-mediated
recombination at Lox P sites generates a site-specific
deletion which leaves only one copy of Lox P in the genome.
Sauer and Henderson, *supra*. This method has been highly
5   successful. Studies assaying the deletion of polymerase β
have reported up to 95% deletion in tissues such as liver,
and as much as 60% deletion, even in non-dividing tissues
such as heart. Kühn, R., *et al.* (1995) *Science 269*:1427-
1429. Thus a mouse can be generated wherein one allele of
10 mTR is deleted, and the second allele is flanked by Lox P
sites. Knockout mTR (-/-) tissues are then generated by
the expression of Cre in specific tissues.

        Although mice which ubiquitously express Cre can be
generated, tissue specific expression is more useful.
15 Several techniques can be used to produce differential
expression of mTR or another telomerase component. Cre can
be placed behind a tissue specific promoter to generate
specific deletion in a given tissue. Gu, H., *et al.* (1994)
Science 265:103-106. Some promoters produce low level
20 recombination and occasional leaky expression in unrelated
tissues using this technique. To avoid these problems, Cre
can be placed behind a tissue specific promoter and fused
to a ligand-binding domain such as the domain from the
estrogen receptor. Picard, D. (1993) *Trends Cell Biol.*
25 *3*:278-280; Logie, C. and Stewart, F. (1995) *Proc. Natl.
Acad. Sci. 92*:5940-5944. Fusion to ligand-binding domains
results in an inactive protein until the appropriate ligand
is added to release the inhibitory effect of the ligand-
binding domain. Picard, D., *et al.* (1988) *Cell 54*:1073-
30 1080. Tissue specific expression can then be induced by
feeding the mice the appropriate receptor ligand. Although
the estrogen receptor has been used for many studies *in
vitro*, the presence of estrogen *in vivo* can activate this
receptor. Thus mutant receptors can be used which respond
35 to artificial, non-physiological drugs.

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Example 8

Tumor Induction in Telomerase-deficient mice

Rates of tumor formation in four different mouse tumor
progression models, RIP-Tag2 insulinomas, K14-HPV squamous
5   cell carcinoma, Eμ-Myc lymphomas and p53 null mice can be
determined. Mice which are homozygous null (-/-), wildtype
(+/+) and heterozygous (+/-) for mTR are crossed to
transgenic mice with high rate of tumor formation.

     a)   Pancreas and skin tumor models.
10   Telomerase expression in two mouse tumor models (RIP-Tag2
and K14-HPV16) has been extensively characterized. RIP-
Tag2 mice and K14-HPV16 mice are crossed with mice
heterozygous for mTR (+/-). Because genetic background
affects the rate of tumor induction in mice, the mTR mice
15   that are in a mixed background of 129 and C57B1/6J are
backcrossed to the appropriate strain to produce a more
homogeneous genotype prior to crossing with mouse tumor
models.
     For the study of RIP-Tag2 induced insulinomas, mTR
20   (+/-) mice are crossed into the C57B1/6 strain, after which
mTR (+\-) females are crossed to RIP-TAg2 males. It is not
possible to introduce the RIP-TAg2 oncogene from the female
side because diabetes is induced by the oncogene
expression. Thus, for this model, the cross is made in
25   only one direction. Pups from this cross are genotyped and
mTR (+/-) males carrying the RIP-Tag2 construct identified.
These mice are subsequently mated to mTR (+/-) females.
Approximately 12.5% of the pups from this cross should be
mTR (-/-) and carry RIP-Tag2. These mice can be identified
30   through Southern blots of tail DNA. Rates of tumor
formation, size of tumors and number of metastases in
litter mates that are mTR (+/+), (+/-) or (-/-) are then
determined. If the numbers of the mice with the
appropriate genotype are low in some litters, siblings from

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identical crosses can be used as controls.

　　　Similar experiments can be carried out to examine the
rate of tumor formation in the K14-HPV16 squamous cell
carcinoma model mice.　The mTR (+/-) mice are backcrossed
5　to FVB/n mice (Arbeit, J.M., et al. (1994) *J. Virol.*
68:4358-4368).　Both male and female mTR (+/-) progeny are
then crossed to K14-HPV expressing mice to generate mice
carrying both mTR (+/-) and the K14-HPV transgene.　Two
heterozygotes carrying K14-HPV can then be crossed and
10　Southern blots used to identify mTR (-/-) mice carrying the
K14-HPV transgene.

　　　In both the RIP-Tag2 and K14-HPV mouse models,
telomerase activity appears to be present in the late
stages of tumor progression, although telomerase RNA is
15　upregulated early.　Blasco, M., et al. (1996) *Nature*
Genetics 12:200-204.　However, not all tumors are
telomerase positive, suggesting that telomerase is not
absolutely required for progression to late stage tumors.
Tumor progression is a stochastic process, not all cells
20　which express the oncogenes become hyperproliferative and
not all hyperplasias progress to tumor formation.　If
telomerase is required for those tumors where it is
expressed, a comparison of mTR (-/-) to mTR (+/+) mice
should show a reduced frequency of tumor formation in the
25　mTR (-/-) mice.

　　　If telomeres in mice are sufficiently long, telomere
length may not be limiting for the number of divisions
required to form a tumor.　There is evidence that, unlike
the tissue culture model where telomeres first shorten to a
30　critical length, telomerase induction in tumors *in vivo* may
not require critical telomere shortening.　Mehle, C., et
al. (1994) *Cancer Res. 54*:236-241.　If no difference in
tumor rates are found, mice with shorter telomeres can be
generated and the experiment repeated using these mice.
35　　　Three different approaches can be taken to generate

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mice with shortened telomeres. First, if the mTR (-/-)
mice are fertile, mice with shortened telomeres can be
generated in a second generation mTR (-/-) mouse. Second,
mTR (+/-) mice can be examined for telomere shortening. It
5   is possible that half of the level of mTR is not sufficient
for telomere maintenance and, consequently, telomere
shortening results. If this occurs, the heterozygous mTR
(+/-) mice can be used as a source of mice with pre-
shortened telomeres. Finally, mice can be generated from
10   mTR (-/-) ES cells that have been grown in culture for
extended periods to allow telomere shortening. mTR (-/-)
ES cells are generated by re-targeting the wildtype allele
in the mTR (+/-) ES cells described supra. A vector
similar to that shown in Figure 4 can be constructed which
15   carries a hygromycin resistance gene in place of the mTR
coding regions. ES cells can be electroporated and
selected for growth in hygromycin. The doubly targeted
cells are identified on Southern blots as described supra.

        b) Lymphoma induction in mTR (-/-) mice.
20   Telomerase activity is present in lymphocytes in both
humans and mice. In human B and T cell malignancies,
telomerase activity is present at higher levels than in
normal cells. Counter, C.M., et al. (1995) Blood 85:2315-
2320; Broccoli, D., et al. (1995) Proc. Natl. Acad. Sci.
25   92:9082-9086. To determine if telomerase activity is
required for induction of lymphomas, lymphoma induction in
Eμ-myc expressing mice (Jackson Laboratories, Bar Harbor,
ME) with or without mTR can be compared. Eμ-myc mice
express the c-myc gene behind an immunoglobin enhancer.
30   These mice reproducibly develop pre-B cell lymphoma within
a few months of birth. Adams, J.M., et al. (1985) Nature
318: 533-538. Crosses with mTR (+/-) mice can be used to
generate Eμ-myc expressing, mTR (+/-) heterozygotes. The
rapidity with which lymphomas develop in this model can

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allow a tumor-suppressor phenotype to be identified in mTR
(+/-) and (-/-) mice. Thus the generation of lymphomas in
genetically identical litter mates that are mTR (+/+) or
(+/-) can be compared. Finally, although tumors are
5 generated at early ages in Eμ-myc expressing mice, it has
been possible to generate males that will live to sexual
maturity and to cross these males. Elson, A., et al.
(1995) *Oncogene 11*:181-190. Eμ-myc male, mTR (+/-) mice
can be crossed to mTR (+/-) females to generate homozygous
10 mTR (-/-) mice expressing Eμ-myc. The onset of lymphoma
development and viability of the mice can then be
monitored. If telomerase is essential for the long-term
survival of B cells, lymphoma development may be retarded
although immune dysfunction may still occur due to the loss
15 of B cells. The lymphoma cells from Eμ-myc mice are easily
cultured *in vitro*. Schmidt, E.V., et al. (1988) *Proc.
Natl. Acad. Sci. 85*:6047-6051; Adams, J.M., et al. (1985)
Nature 318:533-538. Pre-B cells will be cultured from the
lymphomas in these animals and telomere length and the *in
20 vitro* life span of Eμ-myc expressing mTR (+/+), (+/-) or (-
/-) cells can be compared.

c) Effect of p53 deletion in mTR (-/-) mice.
The p53 gene is a tumor suppressor gene which is
frequently mutated in a wide variety of human cancers.
25 Hollstein, M., et al. (1991) *Science 253*:49-53. Mice
heterozygous for p53 develop tumors by around 16 months of
age. Most of these tumors have suffered the loss of the
wildtype allele of p53. Greater than 90% of mice with a
complete (-/-) germline deletion of p53 develop tumors by
30 3-6 months of age. Donehower, L.A., et al. (1992) *Nature
356*:215-221; Jacks, T., et al. (1994) *Curr. Biol. 4*:1-7.
To determine if the absence of telomerase decreases the
tumor incidence or tumor size, mice doubly deficient for
p53 and mTR can be generated. mTR (+/-) mice can be mated

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to p53 (+/-) mice and the tumor incidence and the tumor
spectrum of the progeny examined for both litter mates and
siblings with the following genotypes: mTR (+/+) p53
(+/+), mTR (+/+) p53 (+/-), mTR (+/+) p53 (-/-), mTR (+/-)
5   p53 (+/-), mTR (+/-) p53 (-/-) and mTR (-/-) p53 (-/-).
Since many human tumors are p53 negative, the affect of
telomerase deletion on p53 null cells has important
clinical implications. p53 null cells exhibit dramatic
genomic instability. See, Lane, D.P. (1992) *Nature* 358:15-
10   16. Telomere loss is also predicted to lead to genomic
instability; the combination of telomerase and p53 loss may
lead to cell death. If mTR (-/-) p53 (-/-) mice have
reduced tumor incidence, telomerase inhibitors could be
useful for treatment of p53-tumors in humans. Currently,
15   p53 tumors are the most resistant tumors to chemotherapy.

     d)   mTR deletion in recombination deficient mice.
          In the tumor induction models described above,
telomerase negative tumors could survive due to telomere
elongation via a recombination pathway. To date, no mice
20   have been generated with a germline deficiency in
recombination. In yeast, the Rad 52 recombination pathway
is the best candidate for a potential mediator or telomere
recombination because this pathway is essential for
telomerase bypass in yeast. Lundblad, V. and Blackburn,
25   E.H. (1993) *Cell* 73:347-360. Preliminary evidence suggests
that deletion of the mouse Rad 52 gene is lethal. However,
other genes in the same pathway could also allow
recombinational bypass of telomerase deletion. The mouse
homologues of Rad54 and Rad 51 have been cloned and Rad 54
30   (-/-) ES cells have been produced that are viable and
recombination deficient. If either the Rad 54 or Rad 51 (-
/-) mice are viable or if the heterozygotes show reduced
recombination, these animals can be crossed to mTR (+/-)
mice to generate recombination deficient mTR (-/-) mice.

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Fibroblasts from these cells can be transformed with Tag
and ras, and tested for their ability to form tumors in
nude mice as described *supra*. Ultimately, tumor induction
in the transgenic models described above can be examined to
5 determine if recombination deficiency reduces the growth or
formation of potential telomerase negative tumors.

<u>Equivalents</u>

    Those skilled in the art will recognize, or be able to
ascertain using no more than routine experimentation, many
10 equivalents to the specific embodiments of the invention
described specifically herein. Such equivalents are
intended to be encompassed in the scope of the following
claims.

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Claims

We claim:

1.   A nonhuman transgenic organism wherein at least one
     endogenous gene encoding a telomerase component is
5   altered and telomerase activity is altered.

2.   The organism of Claim 1 wherein the endogenous gene
     encodes all or part of the telomerase RNA component.

3.   A cell or tissue obtained from the organism of Claim
     1.

10 4.   The organism of Claim 1 wherein the expression of
     telomerase activity is reduced as compared to a
     wildtype organism of the same species.

5.   A cell or tissue of the organism of Claim 4.

6.   A nonhuman transgenic organism wherein pPNT-mTRΔ is
15   incorporated into the germline cells at the mTR locus.

7.   A somatic or germline cell of a nonhuman animal
     comprising an exogenous telomerase gene sequence.

8.   A germline cell according to Claim 7 which is an
     embryonic stem cell.

20 9.   The organism of Claim 1 wherein the endogenous gene
     encoding a telomerase component is altered through
     insertion into the genome of the organism of a nucleic
     acid sequence comprising at least a portion of an
     isogenic coding sequence linked to a marker sequence.

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10. The marker sequence of Claim 9 that is a neomycin
     resistance gene.

11. A cell or tissue of the organism of Claim 9.

12. A nucleated cell of the organism of Claim 9 wherein
     the cell is selected from the group consisting of
     embryonic stem cells, embryonic fibroblasts, germline
     cells, somatic cells, white blood cells, and liver
     cells.

13. A nonhuman transgenic organism wherein an endogenous
     gene encoding a telomerase component, which is
     normally in the corresponding nonhuman wildtype
     organism, is not expressed.

14. The nonhuman transgenic organism of Claim 13 which is
     homozygous for an exogenous DNA sequence that replaces
     all or a part of an endogenous gene encoding the RNA
     component of telomerase.

15. The nonhuman transgenic organism of Claim 13 which
     contains no endogenous gene sequence capable of
     expressing functional telomerase.

16. The organism of Claim 13 which contains only one
     endogenous gene sequence per cell capable of
     expressing functional telomerase compared to the
     genome of a corresponding cell of a wildtype organism
     of the same species.

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17. A nucleic acid probe which can detect the length of a
    telomerase gene fragment, wherein the length of the
    fragment determines the capability of a telomerase
    gene sequence to encode a functional telomerase RNA
5   component.

18. A DNA construct comprising DNA encoding exogenous DNA
    between the 3.3 kb XbaI fragment of the 5' end of the
    mouse telomerase RNA component gene and the 4.0 kb
    XhoI fragment of the 3' end of the mouse telomerase
10 RNA component gene.

19. The construct of Claim 18 wherein the exogenous DNA is
    a marker gene sequence.

20. The construct of Claim 19 wherein the marker sequence
    is a neomycin resistance gene.

15 21. The construct of Claim 18 further comprising a
       promoter.

22. The construct of Claim 21 wherein the promoter is an
    inducible promoter.

23. The construct of Claim 22 wherein the inducible
20 promoter is a tetracycline-responsive cytomegalovirus
    promoter.

24. A mouse embryonic stem cell containing the DNA
    construct of Claim 18.

25. A plasmid comprising pPNT-mTRΔ or its functional
25 equivalent.

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26.   A mouse embryonic stem cell selected from the group
       consisting of Tel-1, Tel-2, Tel-3 and Tel-4.

27.   The transgenic nonhuman organism of Claim 1 wherein
       transcription of an endogenous telomerase RNA
5   component sequence is under the control of a promoter
       sequence different from the endogenous promoter
       sequence controlling the transcription of the
       endogenous gene.

28.   A knockout mouse wherein the endogenous gene is
10   replaced in part or in whole by homologous
       recombination between the endogenous gene and a DNA
       construct comprising DNA encoding exogenous DNA
       between the 3.3 kb *Xba*I fragment of the 5' end of the
       mouse telomerase RNA component gene and the 4.0 kb
15   *Xho*I fragment of the 3' end of the mouse telomerase
       RNA component gene.

29.   A method of identifying an agent that stimulates
       telomerase activity, the method comprising:
       a)   administering an agent to a transgenic nonhuman
20   organism, wherein the organism does not express
             telomerase activity or has diminished telomerase
             activity but is capable of producing telomerase
             activity; and
       b)   assessing the effect of the agent on the
25   telomerase activity,
       wherein if the agent causes an increase in telomerase
       activity, the agent is active in stimulating
       telomerase activity.

30.   A method of identifying an agent that stimulates
30   telomerase activity, the method comprising:
       a)   administering an agent to a sample of transgenic

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cells or tissue obtained from a transgenic
nonhuman organism, wherein the sample does not
express telomerase activity or has diminished
telomerase activity but is capable of producing
5   telomerase activity; and
   b)   assessing the effect of the agent on the
          telomerase activity,
wherein if the agent causes an increase in telomerase
activity, the agent is active in stimulating
10   telomerase activity.

   31.   A method of identifying an agent that is active in
         inhibiting telomerase activity, which comprises:
         a)   administering an agent to a transgenic nonhuman
                organism, wherein the organism expresses
15   telomerase activity; and
         b)   assessing the effect of the agent on the
                telomerase activity,
      wherein if the agent causes a decrease in telomerase
      activity, the agent is active in inhibiting telomerase
20   activity.

   32.   A method of identifying side effects of an agent that
         modifies telomerase activity in an organism, which
         comprises:
         a)   administering the agent to a transgenic nonhuman
25   organism which is incapable of expressing
                telomerase; and
   b)   assessing the effects of the agent on the
                transgenic nonhuman organism,
      wherein if the agent causes one or more effects on the
30   organism, it is identified as an agent that causes one or
      more side effects.

TARGETING CONSTRUCT FOR KNOCKING OUT MOUSE TELOMERASE RNA COMPONENT
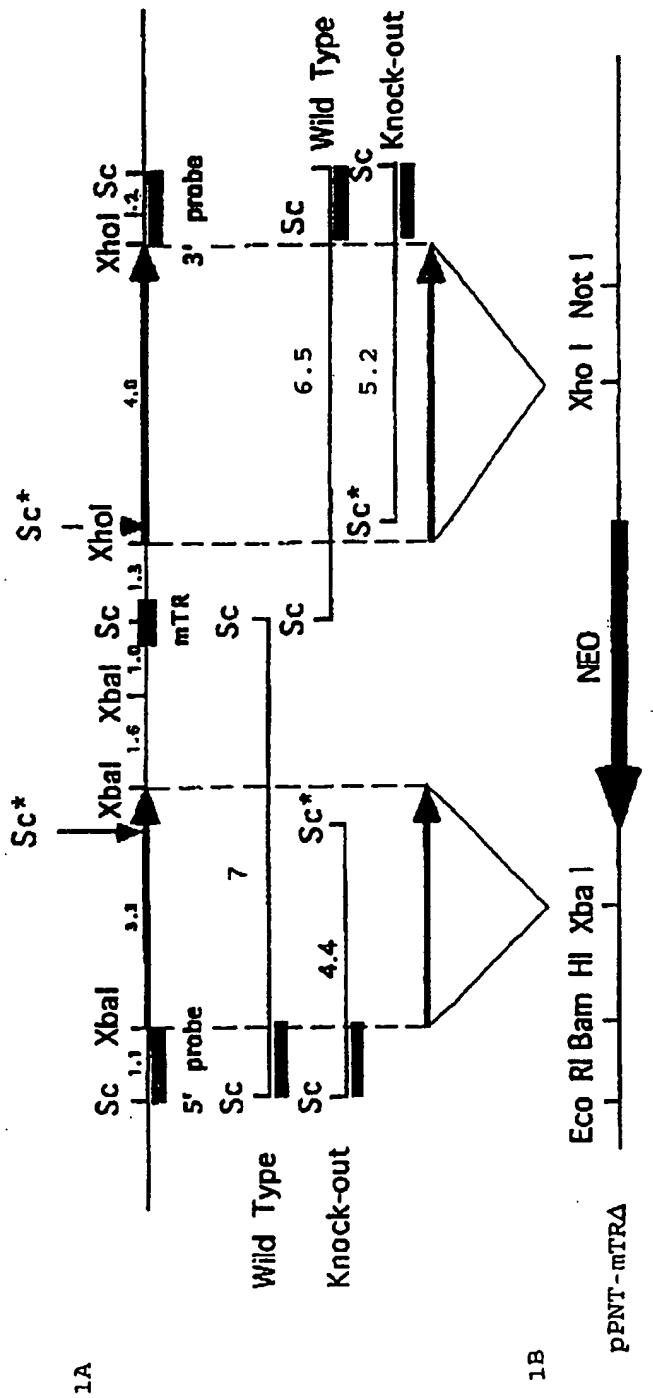
FIGURE 1A-1B

* Site-directed mutagenesis
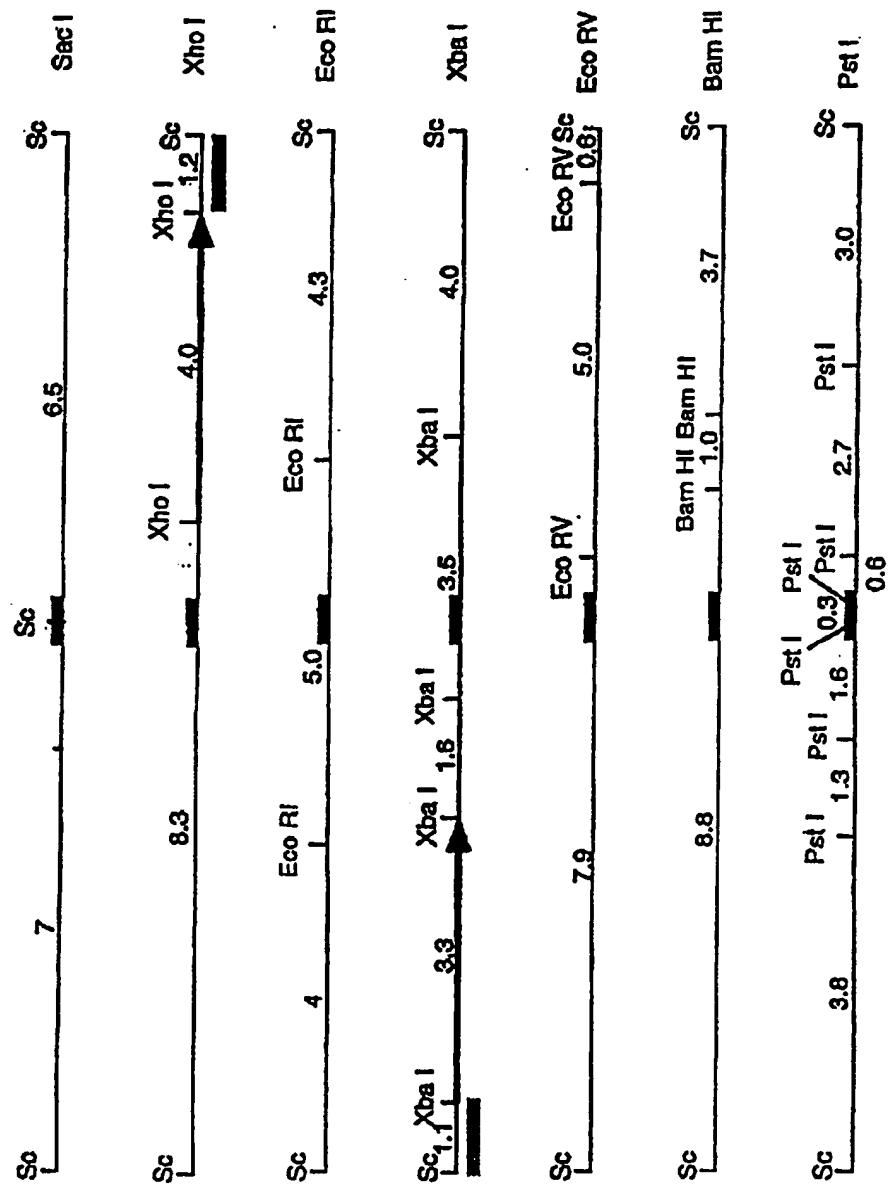
Restriction Map of Genomic mTR gene.

FIGURE 2

CLONING OF XBA I IN SK (-)
(determine orientation)

CLONING OF XHO I IN KS (+)
(determine orientation)

SEQUENCE 3' OF XBA I FRAGMENT
(design primer for mutagenesis)

SEQUENCE 5' OF XHO·I FRAGMENT
(design primer for mutagenesis)

INTRODUCE SAC I RESTRICTION SITE
IN BOTH FRAGMENTS :


201 bp

213 bp
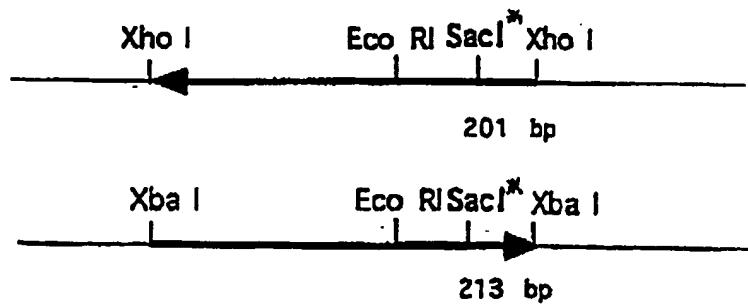
FIGURE 3

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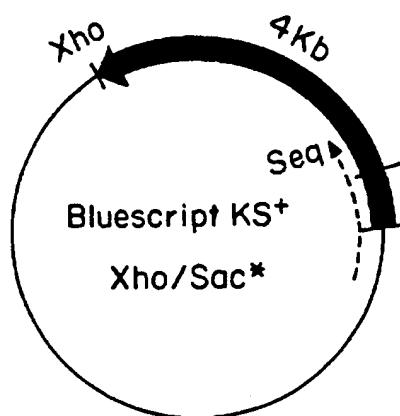
FIG. 4A
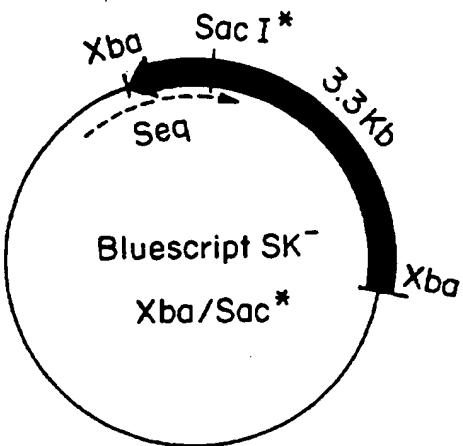
FIG. 4B
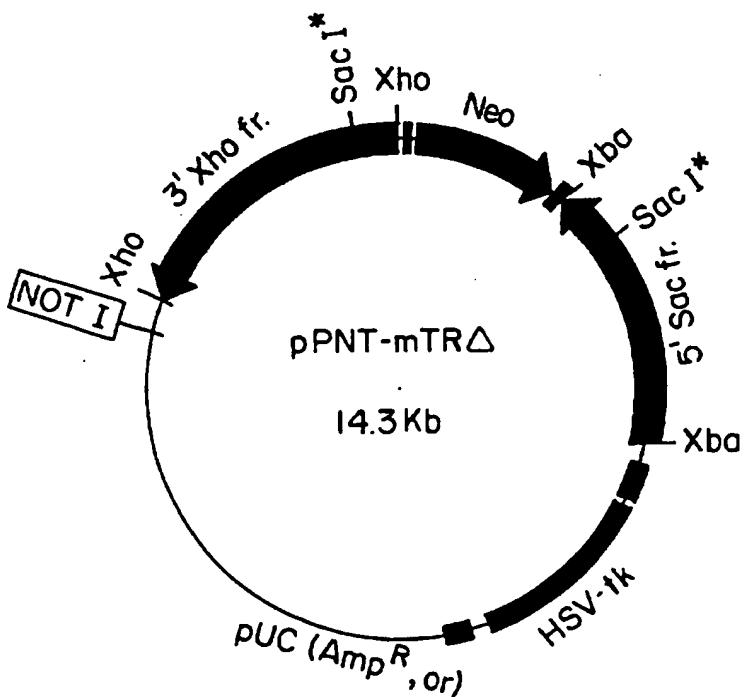
FIG. 4C

Human Telomerase RNA Component Gene

```
-->ST
|
GGGTTGCGGA  GGGTGGGCCT  GGGAGGGGTG  GTGGCCATTT  TTTGTCTAAC   50

CCTAACTGAG  AAGGGCGTAG  GCGCCGTGCT  TTTGCTCCCC  GCGCGCTGTT  100

TTTCTCGCTG  ACTTTCAGCG  GGCGGAAAAG  CCTCGGCCTG  CCGCCTTCCA  150

CCGTTCATTC  TAGAGCAAAC  AAAAAATGTC  AGCTGCTGGC  CCGTTCGCCT  200

CCCGGGGACC  TGCGGCGGGT  CGCCTGCCCA  GCCCCCGAAC  CCCGCCTGGA  250

GCCGCGGTCG  GCCCGGGGCT  TCTCCGGAGG  CACCCACTGC  CACCGCGAAG  300

AGTTGGGCTC  TGTCAGCCGC  GGGTCTCTCG  GGGGCGAGGG  CGAGGTTCAC  350

CGTTTCAGGC  CGCAGGAAGA  GGAACGGAGC  GAGTCCCGCC  GCGGCGCGAT  400
                                              |>STP
TCCCTGAGCT  GTGGGACGTG  CACCCAGGAC  TCGGCTCACA  CATGCAGTTC  450

GCTTTCCTGT  TGGTGGGGGG  AACGCCGATC  GTGCGCATCC  GTCACCCCTC  500

GCCGGCAGTG  GGGGCTTGTG  AACCCCCAAA  CCTGACTGAC  TGGGCCAGTG  550

TGCTGCAAAT  TGGCAGGAGA  CGTGAAGGCA  CCTCCAAAGT  CGGCCAAAAT  600

GAATGGGCAG  TGAGCCGGGG  TTGCCTGGAG  CCGTTCCTGC  GTGGGTTCTC  650

CCGTCTTCCG  CTTTTTGTTG  CCTTTTATGG  TTGTATTACA  ACTTAGTTCC  700

TGCTCTGCAG  ATTTTGTTGA  GGTTTTTGCT  TCTCCCAAGG  TAGATCTCGA  750

CCAGTCCCTC  AACGGGGTGT  GGGGAGAACA  GTCATTTTTT  TTTGAGAGAT  800

CATTTAACAT  TTAATGAATA  TTTAATTAGA  AGATCTAAAT  GAACATTGGA  850

AATTGTGTTC  CTTTAATGGT  CATCGGTTTA  TGCCAGAGGT  TAGAAGTTTC  900

TTTTTTGAAA  AATTAGACCT  TGGCGATGAC  CTTGAGCAGT  AGGATATAAC  950

CCCCACAAGC  TT                                             962
```

FIGURE 5

—

Mouse Telomerase RNA Component Gene

```
CTCGACCAAT  CAGCGCGCGC  CATGGGGTAT  TTAAGGTCGA  GGGCGGCTAG    50
GCCTCGGCAC  CTAACCCTGA  TTTTCATTAG  CTGTGGGTTC  TGGTCTTTTG   100
TTCTCCGCCC  GCTGTTTTTC  TCGCTGACTT  CCAGCGGGCC  AGGAAAGTCC   150
AGACCTGCAG  CGGGCCACCC  GGCGTTCCCG  AGCCTCAAAA  ACAAACGTCA   200
GCGCAGGAGC  TCCAGGTTCG  CCGGGAGCTC  CGCGGCGCCG  GGCCGCCCAG   250
TCCCGTACCC  GCCTACAGGC  CGCGGCCGGC  CTGGGGTCTT  AGGACTCCGC   300
TGCCGCCGCG  AAGAGCTCCG  CCTCTGTCAG  CCGCGGGCGC  GCGGGGGCTG   350
GGGCCAGGCC  GGGCGAGCGC  CGCGAGGACA  GGAATGGAAC  TGGTCCCCGT   400
GTTCGGTGTC  TTACCTGAGC  TGTGGGAAGT  GCACCCGGAA  CTCGGTTCTC   450
ACAACCCCCA  TTCCCGCTGG  GGAAATGCCC  CGCTGCAGGG  CGGGCCGCTA   500
GAACCTGCGA  CTCTGGGGAA  AGGGGCTTCG  GTGTGAGACG  GTAGCCAGCC   550
AAAGGGTATA                                                  560
```

FIGURE 6

```
                                                      +1
hTR   GCTCCCTTTATAAGCCGACTCGCCCGGCAGCGCACCGGGTTGCGGAGGGA
bTR   CAGCCTTCAAAAATGAGGAGCATCCCGGGTTGCGGAGGGTGGGCCCCGGGTT
cTR   GCGAGAGCCGGCGCCGGCCAATCAGCGCGCGCCACC--------------CC
mTR   GCAAGTGCTGGGCTCGACCAATCAGCGCGCGCCATGGGGTATTTAAGGGTC
rTR   TGACTATTAGGGCTCAGCCAATCAGCGCGAGCTGTCGGGTATTTAGGGAC

hTR   GGGTGGGCCTGGGGAGGGGTGGTGGC----CATTTTTGTCTAACCCTGAA-
bTR   GGGTGGGCCC---CGGGTTGGTGGCAGCCATTTCTCATCTAACCCTGAAT
cTR   GGGTACTTAAGGGCGACCTGGCGGGC-GGCT-GCCAGTCTAACCCTGAAT
mTR   GGGTATTTAAGGTCGA--GGGCGGCTAGGCCTCGGCACCTAACCCTGATT
rTR   GGGTATTTAGGGACAA--GGGCCGCGCGACTTCTGCGTCTAACCCTGATT

hTR   -CTGAGAAGGGCGGTAGGCGCCGTGCTTTGCTCCCCGGCGCTGTTTTT
bTR   T--GAGACAGGCGGTAGGCGCTGTGCTTTGGTTACCGGCCGCTGTTTTT
cTR   TCTGAG---AGCGGTGGGTACTGTGCTTTCGGCTCCGGCGCTGTTTTT
mTR   TTCATT---AGCGGTGGGTCTGGTCTTTTGTGCTCCGGCGCTGTTTTT
rTR   GTTATA---GCGGTGGGTTCTGTTCTTTTGTGCTCCGGCGCTGTTTTT

hTR   CTCGCTGACTTTCAGCGGGCC-GGAAAAGCCTCGGCCTGCTGGCTTCCACC
bTR   CTCGCTGACTTTCAGCGGGCC-GGAAAAGCCTCGGCCTACGCCATCCACC
cTR   CTCGCTGACTTCCAGCGGGCC-GGGAAAGTCCAGACCTGCAGCGGGCCATC
mTR   CTCGCTGACTTCCAGCGGGCCAGGAAAGTCCAGACCTGCAGCGGGCCACC
rTR   CTCGCTGACTTCCAGCGGGCCTGGAAAGTTCAGACCTGCAGCGGGTCACC

hTR   GTTCATTCTAGACAAACAAAAAA----TGTCAGCTGCTGGCCGTTCGCC
bTR   ATCCAGTCTGCAACAAACAAAAAA----TGTCAGCCGCTGGCTGCTCACC
cTR   GCGCGTTTTCCACCA-CAAAAAAA--TGTCAGCGCTGGCGTCATGTGCC
mTR   CGGCGTTCCCGAGCCT-CAAAAACAAACGTCAGCGCAAGGAGCTCCAGGT
rTR   GCGCATTCTGGACCT-CAAAAAA----TGTCAGCGTAGGAAGTC-TGGT

hTR   CCTCCCGGGGACTGCGGCGGGT---CGCTGCCCAGCCCCCGAACCCCGCC
bTR   TCTCCCGGGACCTGCGGTGGT-CCGCCGCCCAGCCCCAGTGCCCCGCC
cTR   T-------GGACCT----TGCG-CCGGCGCGCCAGCCCCGCA--CCCGCC
mTR   TCGCCG-GGACCTTCCGCGCGCCGGGCCGCCCAGTCCCGTA--CCCGCC
rTR   GCC-----AGACCTTCCGCGCGCGCTGGGCCGCCAGCCCGGTA--CCCGCC

hTR   TGGAGGCCGCGGTCGGCC-GGGCTTCTCGGAGGCACCCACTGCCACCG
bTR   TGG-AGGCCGCGGTCGGCC-GGGCTTCTCCGGAGGCACCCATTGCCGCCG
cTR   TGG-AGGCCGCGGTCGGC-TGGAG-----TCCTCGGGCTCC-GCTGCCGCCG
mTR   TACAGGCCGCGGCCGGCTGGG-----TCTTAGGACTC-GCTGCCGCCG
rTR   TGGAGGCCGCGGACGGCTGGG-----TCTTAGAACTC-GCTGCCGCCG

hTR   CGAAGAGTTGGCTCTGTCAGCCGCGGGTCTCTCGGGGGCGAGGCGAG
bTR   TGAAGAGTTGGCTCTGTCAGCCGCGGGTCGCTCGGTGGGCCGAGGCAT
cTR   CGAAGAGTTAGACTCTGTCAGCCGCGGG-GCGTCAGGGGCTGGGCGA-
mTR   CGAAGAGTTCGCCTCTGTCAGCCGCGGG-CGCGCGGGGGCTGGGCCAG
rTR   TGAAGAGTTAGTCTCTGTTAGCTACGGG-GCACCGGGGCTGGGGTCAG
```

FIGURE 7A

```
hTR  GTTCACCGTTTCAGGCCGC--AGGAAGAGGAACGGAGCGA-GTCCC-GCG
bTR  GGCTGTA--------ACGC-AGGGAAAGGAACGGAGTGGGGTCCCCGCG
cTR  GCCGG--CAGC----GCGGCAAGCAGAGAAA-CGGAGCTG-GTCCC-GTG
mTR  GCCGGGCGAGC----GCGCGAGGACAGGAAT-GGAACTG-GTCCCCGTG
rTR  GCCGGGAGAGC----GCGGCAAGGACAGTAAC-GGAACTG-GTCCCTGAG

hTR  CGCGGCGCGATCTCCTGAGCTATGGGACGTGCACCCAGGACTCGGCTCAC
bTR  CGCG-TGCG-TTCCCTGAGCTGTGGGACTTGCACCCGGGACTCGGCTCAG
cTR  AACGGTAAC-TTCCCTGAGTTGTGGGAAATGCACCAGGAACTCGGTCCC
mTR  TTCGGTGTC-TTACCTGAGCTGTGGGAAGTGCACCCGGAACTCGGCTCTC
rTR  TTCGGTGGC-TTTCCTGAGATGTGGGAAGTGCACCTGGAACTCGGTTCCT

hTR  ACATGCAGTTCGCTTTCCTGTTGGTGGGGGAACGGCGATCGTGCGCATC
bTR  ACATCTGAAAAAAAAAAAAAA-----TGAGGAGAT-CCTACCATATG-AAAC
cTR  ACAACCCCCAACCCGC--------TGGGAAATAACGTG-CTGCAAAGCGG
mTR  ACAACCCCCATTCCGC--------TGGGAAATGCCCGCTGCAGGGCGG
rTR  ACAACCCCCACTTCCGC--------TGGGAAA-TGCGTTGCTACCTGGCGG

hTR  CGTCACCCCTCGCCGGCAGTGGGGGCTTGTGAACCCCCAAACCTGACTGA
bTR  AATATGAAC-AAAACTTG-AGGTTGTGCTAAGTGAAG-TAAGTC-------
cTR  .G----CCCCTAGGACCTGGCAGCCCGAGGAATGGTG-CCAACGTGTGTGC
mTR  G----CCGCTAGAACCTG-CGACTCTGGGGAAAGGGGCTTCGGTGTGAGA
rTR  GG----CGCTAGAAC-TG-CAACCGGGAGGAACGGGGCCAAGGTGTGTGC

hTR  CTG-GGCCAGTGTGCTGC
bTR  ----AGCCATAGAAGGACAAATACTGTTACAATTC
cTR  ACATGGCCAGAGTGGGCGATG
mTR  CGGTAGCCAGCCAAAGGGTATA
rTR  ACGAGGCCACGGTGCTC
```

FIGURE 7B

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Tetrahymena 80kD Telomerase Protein Component Gene

```
   1 aactcattta attactaatt taatcaacaa gattgataaa aagcagtaaa taaaacccaa
  61 tagatttaat ttagaaagta tcaattgaaa aatggaaatt gaaaacaact aagcacaata
 121 gccaaaagcc gaaaaattgt ggtgggaact tgaattagag atgcaagaaa accaaaatga
 181 tatataagtt agggttaaga ttgacgatcc taagcaatat ctcgtgaacg tcactgcagc
 241 atgtttgttg taggaaggta gttactacta agataaagat gaaagaagat atatcatcac
 301 taaagcactt cttgaggtgg ctgagtctga tcctgagttc atctgctagt tggcagtcta
 361 catccgtaat gaactttaca tcagaactac cactaactac attgtagcat tttgtgttgt
 421 ccacaagaat actcaaccat tcatcgaaaa gtacttcaac aaagcagtac ttttgcctaa
 481 tgacttactg gaagtctgtg aatttgcata ggttctctat attttttgatg caactgaatt
 541 caaaaatttg tatcttgata ggatactttc ataagatatt cgtaaggaac tcactttccg
 601 taagtgttta caaagatgcg tcagaagcaa gttttctgaa ttcaacgaat actaacttgg
 661 taagtattgc actgaatcct aacgtaagaa aacaatgttc cgttacctct cagttaccaa
 721 caagtaaaag tgggattaaa ctaagaagaa gagaaaagag aatctcttaa*ccaaacttta
 781 ggcaataaag gaatctgaag ataagtccaa gagagaaact ggagacataa tgaacgttga
 841 agatgcaatc aaggctttaa aaccagcagt tatgaagaaa atagccaaga gatagaatgc
 901 catgaagaaa cacatgaagg cacctaaaat tcctaactct accttggaat caaagtactt
 961 gaccttcaag gatctcatta agttctgcca tatttctgag cctaaagaaa gagtctataa
1021 gatccttggt aaaaaatacc ctaagaccga agaggaatac aaagcagcct ttggtgattc
1081 tgcatctgca cccttcaatc ctgaattggc tggaaagcgt atgaagattg aaatctctaa
1141 aacatgggaa aatgaactca gtgcaaaagg caacactgct gaggtttggg ataatttaat
1201 ttcaagcaat taactcccat atatggccat gttacgtaac ttgtctaaca tcttaaaagc
1261 cggtgtttca gatactacac actctattgt gatcaacaag atttgtgagc ccaaggccgt
1321 tgagaactcc aagatgttcc ctcttcaatt ctttagtgcc attgaagctg ttaatgaagc
1381 agttactaag ggattcaagg ccaagaagag agaaaatatg aatcttaaag gtcaaatcga
1441 agcagtaaag gaagttgttg aaaaaaccga tgaagagaag aaagatatgg agttggagta
1501 aaccgaagaa ggagaatttg ttaaagtcaa cgaaggaatt ggcaagcaat acattaactc
1561 cattgaactt gcaatcaaga tagcagttaa caagaattta gatgaaatca aaggacacac
1621 tgcaatcttc tctgatgttt ctggttctat gagtacctca atgtcaggtg gagccaagaa
1681 gtatggttcc gttcgtactt gtctcgagtg tgcattagtc cttggtttga tggtaaaata
1741 acgttgtgaa aagtcctcat tctacatctt cagttcacct agttctcaat gcaataagtg
1801 ttacttagaa gttgatctcc ctggagacga actccgtcct tctatgtaaa aacttttgca
1861 agagaaagga aaacttggtg gtggtactga tttcccctat gagtgcattg atgaatggac
1921 aaagaataaa actcacgtag acaatatcgt tattttgtct gatatgatga ttgcagaagg
1981 atattccagat atcaatgtta gaggcagttc cattgttaac agcatcaaaa agtacaagga
2041 tgaagtaaat cctaacatta aaatctttgc agttgactta gaaggttacg gaaagtgcct
2101 taatctaggt gatgagttca atgaaaacaa ctacatcaag atattcggta gagcgattc
2161 aatcttaaag ttcatttcag ccaagcaagg aggagcaaat atggtcgaag ttatcaaaaa
2221 ctttgccctt caaaaaatag gacaaaagtg agtttcttga gattcttcta taacaaaaat
2281 ctcaccccac ttttttgttt tattgcatag ccattatgaa atttaaatta ttatctattt
2341 atttaagtta cttacatagt ttatgtatcg cagtctatta gcctattcaa atgattctgc
2401 aaagaacaaa aaagattaaa a
```

FIGURE 8

Amino Acid Sequence of the _Tetrahymena_
80 kD Telomerase Protein Component

MEIENNQAQQPKAEKLWWELELEMQENQNDIQVRVKIDDPKQYL

VNVTAACLLQEGSYYQDKDERRYIITKALLEVAESDPEFICQLAVYIRNELYIRTTTN

YIVAFCVVHKNTQPFIEKYFNKAVLLPNDLLEVCEFAQVLYIFDATEFKNLYLDRILS

QDIRKELTFRKCLQRCVRSKFSEFNEYQLGKYCTESQRKKTMFRYLSVTNKQKWDQTK

KKRKENLLTKLQAIKESEDKSKRETGDIMNVEDAIKALKPAVMKKIAKRQNAMKKHMK

APKIPNSTLESKYLTFKDLIKFCHISEPKERVYKILGKKYPKTEEEYKAAFGDSASAP

FNPELAGKRMKIEISKTWENELSAKGNTAEVWDNLISSNQLPYMAMLRNLSNILKAGV

SDTTHSIVINKICEPKAVENSKMFPLQFFSAIEAVNEAVTKGFKAKKRENMNLKGQIE

AVKEVVEKTDEEKKDMELEQTEEGEFVKVNEGIGKQYINSIELAIKIAVNKNLDEIKG

HTAIFSDVSGSMSTSMSGGAKKYGSVRTCLECALVLGLMVKQRCEKSSFYIFSSPSSQ

CNKCYLEVDLPGDELRPSMQKLLQEKGKLGGGTDFPYECIDEWTKNKTHVDNIVILSD

MMIAEGYSDINVRGSSIVNSIKKYKDEVNPNIKIFAVDLEGYGKCLNLGDEFNENNYI

KIFGMSDSILKFISAKQGGANMVEVIKNFALQKIGQK"

FIGURE 9

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Tetrahymena 95 kD Telomerase Protein Component Gene

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   1  tcaatactat taattaataa ataaaaaaaa gcaaactaca aagaaaaatgt caaggcgtaa
  61  ctaaaaaaag ccataggctc ctataggcaa tgaaacaaat cttgatttttg tattacaaaa
 121  tctagaagtt tacaaaagcc agattgagca ttataagacc tagtagtaat agatcaaaga
 181  ggaggatctc aagctttaa agttcaaaaa ttaagattag gatggaaact ctggcaacga
 241  tgatgatgat gaagaaaaca actcaaataa ataataagaa ttattaagga gagtcaatta
 301  gattaagtag caagtttaat tgataaaaaa agttggttct aaggtagaga aagatttgaa
 361  tttgaacgaa gatgaaaaca aaaagaatgg actttctgaa tagcaagtga aagaagagta
 421  attaagaacg attactgaag aataggttaa gtattaaaat ttagtattta acatggacta
 481  ccagttagat ttaaatgaga gtggtggcca tagaagacac agaagagaaa cagattatga
 541  tactgaaaaa tggtttgaaa tatctcatga ccaaaaaaat tatgtatcaa tttacgccaa
 601  ctaaaagaca tcatattgtt ggtggcttaa agattatttt aataaaaaca attatgatca
 661  tcttaatgta agcattaaca gactagaaac tgaagccgaa ttctatgcct ttgatgattt
 721  ttcacaaaca atcaaactta ctaataattc ttactagact gttaacatag acgttaattt
 781  tgataataat ctctgtatac tcgcattgct tagattttta ttatcactag·aaagattcaa
 841  tattttgaat ataagatctt cttatacaag aaattaatat aattttgaga aaattggtga
 901  gctacttgaa actatcttcg cagttgtctt ttctcatcgc cacttacaag gcattcattt
 961  acaagttcct tgcgaagcgt tctaatattt agttaactcc tcatcataaa ttagcgttaa
1021  agatagctaa ttataggtat actctttctc tacagactta aaattagttg acactaacaa
1081  agtccaagat tattttaagt tcttataaga attccctcgt ttgactcatg taagctagta
1141  ggctatccca gttagtgcta ctaacgctgt agtgttttac ttaaaaaggt
1201  caagcatgct aatcttaatt tagtttctat ccctacctaa ttcaattttg atttctactt
1261  tgttaatttta taacatttga aattagagtt tggattagaa ccaaatattt tgacaaaaca
1321  aaagcttgaa aatctacttt tgagtataaa ataatcaaaa aatcttaaat ttttaagatt
1381  aaactttttac acctacgttg cttaagaaac ctccagaaaa cagatattaa aacaagctac
1441  aacaatcaaa aatctcaaaa acaataaaaa tcaagaagaa actcctgaaa ctaaagatga
1501  aactccaagc gaaagcacaa gtggtatgaa attttttgat catctttctg aattaaccga
1561  gcttgaagat ttcagcgtta acttgtaagc tacccaagaa atttatgata gcttgcacaa
1621  acttttgatt agatcaacaa atttaaagaa gttcaaatta agttacaaat atgaaatgga
1681  aaagagtaaa atggatacat tcatagatct taagaatatt tatgaaacct taaacaatct
1741  taaaagatgc tctgttaata tatcaaatcc tcatggaaac atttcttatg aactgacaaa
1801  taaagattct actttttata aatttaagct gaccttaaac taagaattat aacacgctaa
1861  gtatactttt aagtagaacg aattttaatt taataacgtt aaaagtgcaa aaattgaatc
1921  ttcctcatta gaaagcttag aagatattga tagtctttgc aaatctattg cttcttgtaa
1981  aaatttacaa aatgttaata ttatcgccag tttgctctat cccaacaata tttagaaaaa
2041  tcctttcaat aagcccaatc ttctattttt caagcaattt gaataattga aaaatttgga
2101  aaatgtatct atcaactgta ttcttgatca gcatatactt aattctattt cagaattctt
2161  agaaaagaat aaaaaaataa aagcattcat tttgaaaaga tattatttat tacaatatta
2221  tcttgattat actaaattat ttaaaacact tcaatagtta cctgaattaa attaagttta
2281  cattaattag caattagaag aattgactgt gagtgaagta cataagtaag tatgggaaaa
2341  ccacaagcaa aaagctttct atgaaccatt atgtgagttt atcaaagaat catcctaaac
2401  cctttagcta ·atagattttg accaaaacac tgtaagtgat gactctatta aaaagatttt
2461  agaatctata tctgagtcta agtatcatca ttatttgaga ttgaaccta gttaatctag
2521  cagtttaatt aaatctgaaa acgaagaaat ttagaactt ctcaaagctt gcgacgaaaa
2581  aggtgtttta gtaaaagcat actataaatt ccctctatgt ttaccaactg gtacttatta
2641  cgattacaat tcagatagat ggtgattaat taaatattag tttaaataaa tattaaatat
2701  tgaatatttc tttgcttatt atttgaataa tacatacaat agtcattttt agtgttttga
2761  atatattttta gttatttaat tcattatttt aagtaaataa ttatttttca atcatttttt
2821  aaaaaatcg
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FIGURE 10

SUBSTITUTE SHEET (RULE 26)

Amino Acid Sequence of the *Tetrahymena*
95 kD Telomerase Protein Component

MSRRNQKKPQAPIGNETNLDFVLQNLEVYKSQIEHYKTQQQQIK
EEDLKLLKFKNQDQDGNSGNDDDDEENNSNKQQELLRRVNQIKQQVQLIKKVGSKVEK
DLNLNEDENKKNGLSEQQVKEEQLRTITEEQVKYQNLVFNMDYQLDLNESGGHRRHRR
ETDYDTEKWFEISHDQKNYVSIYANQKTSYCWWLKDYFNKNNYDHLNVSINRLETEAE
FYAFDDFSQTIKLTNNSYQTVNIDVNFDNNLCILALLRFLLSLERFNILNIRSSYTRN
QYNFEKIGELLETIFAVVFSHRHLQGIHLQVPCEAFQYLVNSSSQISVKDSQLQVYSF
STDLKLVDTNKVQDYFKFLQEFPRLTHVSQQAIPVSATNAVENLNVLLKKVKHANLNL
VSIPTQFNFDFYFVNLQHLKLEFGLEPNILTKQKLENLLLSIKQSKNLKFLRLNFYTY
VAQETSRKQILKQATTIKNLKNNKNQEETPETKDETPSESTSGMKFFDHLSELTELED
FSVNLQATQEIYDSLHKLLIRSTNLKKFKLSYKYEMEKSKMDTFIDLKNIYETLNNLK
RCSVNISNPHGNISYELTNKDSTFYKFKLTLNQELQHAKYTFKQNEFQFNNVKSAKIE
SSSLESLEDIDSLCKSIASCKNLQNVNIIASLLYPNNIQKNPFNKPNLLFFKQFEQLK
NLENVSINCILDQHILNSISEFLEKNKKIKAFILKRYYLLQYYLDYTKLFKTLQQLPE
LNQVYINQQLEELTVSEVHKQVWENHKQKAFYEPLCEFIKESSQTLQLIDFDQNTVSD
DSIKKILESISESKYHHYLRLNPSQSSSLIKSENEEIQELLKACDEKGVLVKAYYKFP
LCLPTGTYYDYNSDRW

FIGURE 11

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GGGCGGATCCATGGAGATCGAGAACAACCAAGCTCAACAACGGAAGGCTGAGAAGCTGTGG
TGGGAGCTGGAGCTGGAGATGCAAGAGAACCAAAACGACATCCAAGTTCGTGTTAAGATCG
ACGACCCGAAGCAATACCTGGTTAACGTTACCGCTGCTTGTCTGCTGCAAGAGGGCAGCTA
CTACCAAGACAAGGACGAGCGGTCGTTACATCATCACCAAGGCTCTGCCTGGAGGTTGCTGAG
AGCGACCCGGAGTTCATCTGTCAACTGGCTGTTTACATCCGTAACGAGCTGTACATCCGTA
CCACCACCAACTACATCGTTGCTTTCTGTGTTGTTCACAAGAACACCCAACCGTTCATCGA
GAAGTACTTCAACAAGGCTGTTCTGCTGCCGAACGACCTGCTGGAGGTTTGTGAGTTCGCT
CAAGTTCTGTACATCTTCGACGCTACCGAGTTCAAGAACCTGTACCTGGACCGTATCCTGA
GCCAAGATATCCGTAAGGAGCTGACCTTCCGTAAGTGTCTGCAACGTTGTGTTCGTAGCAA
GTTCAGCGAGTTCAACGAGTACCAACTGGGCAAGTACTGTACCGAGAGCCAACGTAAGAAG
ACCATGTTCCGTTACCTGAGCGTTACCAACAAGCAAAAGTGGGACCAAACCAAGAAGAAGC
GTAAGGAGAACCTGCTGACCAAGCTGCAAGCTATCAAGGAGAGCGAGGACAAGAGCAAGCG
TGAGACCGGCGACATCATGAACGTTGAGGACGCTATCAAGGCTGTGAAGCCGGCCGTTATG
AAGAAGATCGCTAAGCGTCAAAAACGCTATGAAGAAGCACATGAAGGCTCCGAAGATCCCGA
ACAGCACCCTGGAGAGCAAGTACCTGACCTTCAAGGACCTGATCAAGTTCTGTCACATCAG
CGAGCCGAAGGAACGTGTTTACAAGATCCTGGGCAAGAAGTACCCGAAGACCGAGGAGGAG
TACAAGGCTGCTTTCGGCGACAGCGCTAGCGCTCCGTTCAACCCGGAGCTGGCTGGCAAGC
GTATGAAGATCGAGATCAGCAAGACCTGGGAGAACGAGCTGAGCGCTAAGGGCAACACCGC
TGAGGTTTGGGACAACCTGATCAGCAGCAACCAACTGCCGTACATGGCCATGCTGCGTAAC
CTGAGCAACATCCTGAAGGCTGGCGTTAGCGACACCACCCACAGCATCGTTATCAACAAGA
TCTGTGAGCCGAAGGCTGTTGAGAACAGCAAGATGTTCCCGCTGCAATTCTTCAGCGCTAT
CGAGGCTGTTAACGAGGCGGTTACCAAGGGCTTCAAGGCTAAGAAGCGTGAGAACATGAAC
CTGAAGGGCCAAATCGAGGCTGTTAAGGAGGTTGTTGAGAAGACCGACGAGGAGAAGAAGG
ACATGGAGCTGGAGCAAACCGAGGAGGGCGAATTCGTTAAGGTTAACGAGGGCATCGGCAA
GCAATACATCAACAGCATCGAGCTGGCTATCAAGATCGCTGTGAACAAGAACCTGGACGAG
ATCAAGGGCCACACCGCTATCTTCAGCGACGTCAGCGGCAGCATGAGCACCAGCATGAGCG
GCGGCGCTAAGAAGTACGGCCAGCGTTCGTACCTGTCTCTGGAGTGTGTCTCTGGTTCTGGGCCT
GATGGTTAAGCAACGTTGTGAGAAGAGCAGCTTCTACATCTTCAGCAGCCCGAGCAGCCAA
TGTAACAAGTGTTACCTGGAGGTTGACCTGCCGGGCGACGAGCTGCGTCCGAGCATGCAAA
AGCTGCTGCAAGAGAAGGGCAAGCTGGCGGCGGCACCGACTTCCCGTACGAGTGTATCGA
TGAGTGGACCAAGAACAAGACCCACGTTGACAACATCGTTATCCTGAGCGACATGATGATC
GCTGAGGGCTACAGCGACATCAACGTTCGTGGCAGCAGCATCGTTAACAGCATCAAGAAGT
ACAAGGACGAGGTTAACCCGAACATCAAAATCTTCGCTGTTGACCTGGAGGGCTACGGCAA
GTGTCTGAACCTGGGCGACGAGTTCAACGAGAACAACTACATCAAAATCTTCGGCATGAGC
GACAGCATCCTGAAGTTCATCAGCGCTAAGCAAGGCGGCGCTAACATGGTGGAGGTGATCA
AGAACTTCGCTCTGCAAAAGATCGGCCAAAAGTGACTGCAGACTAGTCTAGAAAGCTTGGT
ACCGCCC
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FIGURE 12

GGGGCCATGGGATGAGCCGTCGTAACCAAAAGAAGCCGCAAGCTCCGATCGGCAACGAGACC
AACCTGGACTTCGTTCTGCAAAACCTGGAGGTTTACAAGAGCCAAATCGAGCACTACAAGA
CCCAACAACAACAAATCAAGGAGGAGGACCTGAAGCTGCTGAAGTTCAAGAACCAAGACCA
AGACGGCAACAGCGGCAACGACGACGACGACGAGGAGAACAACAGCAACAAGCAACAAGAG
CTGCTGCGTCGTGTTAACCAAATCAAGCAACAAGTTCAACTGATCAAGAAGGTTGGCAGCA
AGGTTGAGAAGGACCTGAACCTGAACGAGGACGAGAACAAGAAGAACGGCCTGAGCGAGCA
ACAAGTTAAGGAGGAGCAACTGCGTACCATCACCGAGGAGCAAGTTAAGTACCAAAACCTG
GTTTTCAACATGGACTACCAACTGGACCTGAACGAGAGCGGCGGCCACCGTCGTCACCGTC
GCGAGACCGACTACGACACCGAGAAGTGGTTCGAGATCAGCCACGACCAAAAGAACTACGT
TAGCATCTACGCTAACCAAAAGACCAGCTACTGTTGGTGGCTGAAGGACTACTTCAACAAG
AACAACTACGACCACCTGAACGTTAGCATCAACCGTCTGGAGACCGAGGCTGAGTTCTACG
CTTTCGACGACTTCAGCCAAACCATCAAGCTGACCAACAACAGCTACCAAACCGTTAACAT
CGACGTCAACTTCGACAACAACCTGTGTATCCTGGCTCTGCTGCGTTTCCTGCTGAGCCTG
GAGCGGTTTCAACATCCTGAACATCCGTAGCAGCTACACCCGTAACCAATACAACTTCGAAA
AGATCGGCGAGCTGCTGGAGACCATCTTCGCTGTTGTTTTCAGCCACCGTCACCTGCAAGG
CATCCACCTGCAAGTTCCGTGTGAGGCTTTCCAATACCTGGTTAACAGCAGCAGCCAAATC
AGCGTTAAGGACAGCCAACTGCAAGTTTACAGCTTCAGCACCGACCTGAAGCTGGTTGACA
CCAACAAGGTTCAAGACTACTTCAAGTTCCTGCAAGAGTTCCCGCGTCTGACCCACGTGAG
CCAACAAGCTATCCCGGTTAGCGCTACCAACGCTGTTGAGAACCTGAACGTTCTGCTGAAG
AAGGTTAAGCACGCTAACCTGAACCTGGTTAGCATCCCGACCCAATTCAACTTCGACTTCT
ACTTCGTTAACCTGCAACACCTGAAGCTGGAGTTCGGCCTGGAGCCGAACATCCTGACCAA
GCAAAAGCTGGAGAACCTGCTGCTGAGCATCAAGCAAAGCAAGAACCTGAAGTTCCTGCGT
CTGAACTTCTACACCTACGTTGCTCAAGAGACCAGCCGTAAGCAAATCCTGAAGCAAGCTA
CCACCATCAAGAACCTGAAGAACAACAAGAACCAAGAGGAGACTCCGGAGACCAAGGACGA
GACCCCGAGCGAGAGCACCAGCGGCATGAAGTTCTTCGACCACCTGAGCGAGCTGACCGAG
CTGGAGGACTTCAGCGTTAACCTGCAAGCTACCCAAGAGATCTACGACAGCCTGCACAAGC
TGCTGATCCGTAGCACCAACCTGAAGAAGTTCAAGCTGAGCTACAAGTACGAGATGGAGAA
GAGCAAGATGGACACCTTCATCGATCTGAAGAACATCTACGAGACCCTGAACAACCTGAAG
CGTTGTAGCGTTAACATCAGCAACCCGCACGGCAACATCAGCTACGAGCTGACCAACAAGG
ACAGCACCTTCTACAAGTTCAAGCTGACCCTGAACCAAGGCTGCAACACGCTAAGTACAC
CTTCAAGCAAAACGAATTCCAATTCAACAACGTTAAGAGCGCTAAGATCGAGAGCAGCAGC
CTGGAGAGCCTGGAGGACATCGACAGCCTGTGTAAGAGCATCGCCAGCTGTAAGAACCTGC
AAAACGTTAACATCATCGCTAGCCTGCTGTACCCGAACAACATCCAAAAGAACCCGTTCAA
CAAGCCGAACCTGCTGTTCTTCAAGCAATTCGAGCAACTGAAGAACCTGGAGAACGTTAGC
ATCAACTGTATCCTGGACCAACACATCCTGAACAGCATCAGCGAGTTCCTGGAGAAGAACA
AGAAGATCAAGGCTTTCATCCTGAAGCGTTACTACCTGCTGCAATACTACCTGGACTACAC
CAAGCTGTTCAAGACCCTGCAACAACTGCCGGAGCTGAACCAAGTTTACATCAACCAACAA
CTGGAGGAGCTGACCGTTAGCGAGGTTCACAAGCAAGTTTGGGAGAACCACAAGCAAAAGG
CCTTCTACGAGCCGCTGTGTGAGTTCATCAAGGAGAGCAGCCAAACCCTGCAACTGATCGA
CTTCGACCAAAACACCGTTAGCGACGACAGCATCAAGAAGATCCTGGAGAGCATCAGCGAG
AGCAAGTACCACCACTACCTGCGTCTGAACCCGAGCCAAAGCAGCAGCCTGATCAAGAGCG
AGAACGAGGAGATCCAAGAGCTGCTGAAGGCTTGTGACGAGAAGGGCGTTCTGGTTAAGGC
TTACTACAAGTTCCCGCTGTGTCTGCCGACCGGCACCTACTACGACTACAACAGCGACCGT
TGGTGAGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTCCCGGGAAGCTTGGGG

FIGURE 13